Non-A, Non-B Hepatitis. II. Experimental Transmission, Putative Virus Agents and Markers, and Prevention

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Experimental Transmission

The most compelling evidence for the existence of non-A, non-B (NANB)* hepatitis agents derives from experimental transmission of infection to volunteers and nonhuman primates.

Volunteer Studies

Serologically reevaluating serum samples stored from volunteer studies conducted in the 1950s, Hoofnagle et al. (1) demonstrated that in 9 volunteers inoculated with heterologous hepatitis B surface antigen (HBsAg)-negative serum from 3 donors, NANB hepatitis had developed after incubation periods of 18–89 days. Six of the 9 became jaundiced, and 2 of these 6 had evidence of chronic hepatitis. This study also provided evidence that NANB hepatitis is unrelated to hepatitis B virus (HBV) by demonstrating the absence of anamnestic antibody-to-HBsAg (anti-HBs) responses in recipients immune to HBV after injection with an-NANB inoculum, in contrast to the presence of anamnestic responses in such recipients administered a HBV inoculum. This study also demonstrated that infection with NANB agents confers homologous immunity, for recipients were immune to rechallenge by the same inoculum. Furthermore, because serum used in these transmission experiments was collected from donors 6 mo–1 yr after their original blood donation had been implicated in transmitting hepatitis, this study provided evidence for the existence of chronic NANB hepatitis infection in the donors.

Experimental transmission of NANB hepatitis was reproduced in another study which, in addition, included successful serial passage of an NANB hepatitis agent in volunteers (2). In a 1989 study of blood-borne malaria transmission in prisoner volunteers, 6 persons were subinoculated sequentially, and acute NANB hepatitis developed in the last 4 of 6 in the serial transmission chain. In addition, prospective testing was done in another group of 15 volunteers who received 5 ml of malaria-rich blood from 14 different donors; NANB hepatitis developed in 6.

Many of the characteristic clinical features of NANB hepatitis previously described were observed: both short (2–4 wk) and long (4–8 wk) incubation periods, predominantly asymptomatic illness, and persistent elevation of and fluctuating aminotransferase activity. Moreover, in the serial subinoculation study, the viremia of NANB hepatitis cases appeared to have begun within the first week after inoculation (2). The small size (1–5 ml) of the inocula in these two volunteer studies suggested that transfusion of an entire unit of blood was not necessary to transmit NANB hepatitis, and the fact that the sources of the

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*Abbreviations used in this paper: ALT, alanine aminotransferase; anti-δ, antibody to δ antigen; anti-HAV, antibody to hepatitis A virus; anti-HBc, antibody to hepatitis B core antigen; anti-HBs, antibody to hepatitis B surface antigen; CEA, carcinoembryonic antigen; CEP, counter electrophoresis; CMV, cytomegalovirus; δ, delta; EBV, Epstein–Barr virus; ELISA, enzyme immunoassay; HAV, hepatitis A virus; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; CEA, carcinoembryonic antigen; CMV, cytomegalovirus; δ, delta; EBV, Epstein–Barr virus; ELISA, enzyme immunoassay; HAV, hepatitis A virus; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBV, hepatitis B virus; RNA, hepatitis related antigen; IC, immune globulin; NAB, non-A, non-B; NIH, National Institutes of Health; RF, rheumatoid factor; RIA, radioimmunoassay; TTV Study, Transfusion-Transmitted Viruses Study.
infectious inocula were asymptomatic confirmed the existence of chronic asymptomatic NANB hepatitis carriers.

Chimpanzees

Societal views have changed since these volunteer studies were done, and no such studies are likely to be done in the future. On the other hand, transmission studies of hepatitis viruses in nonhuman primates had been instrumental to advances in hepatitis A virus (HAV) and HBV research, and, shortly after the existence of NANB hepatitis agents was recognized, attempts were made to transmit NANB hepatitis to chimpanzees. To date, after the simultaneous independent reports of successful transmission of NANB hepatitis in chimpanzees in two laboratories (3,4), several other groups of investigators have reported successful, reproducible transmission of human NANB hepatitis to chimpanzees (6-18) and serial passage of the agent(s) in these primates (8,19-28). Such serial passage, and the demonstration that the agent is filterable (5), provide the best evidence that NANB hepatitis is caused by a transmissible agent and that the agent is a virus. Moreover, the chimpanzee has proven to be a valuable animal model that has contributed immeasurably to our knowledge about NANB hepatitis.

A variety of inocula have been used successfully to infect chimpanzees. These include acute-phase and chronic-phase serum or plasma from patients with acute or chronic posttransfusion or community-acquired NANB hepatitis; serum or plasma from blood donors implicated in transmission of hepatitis to blood recipients; clotting factor VIII and factor IX concentrates, as well as fibrinogen; and acute-phase and chronic-phase serum, as well as liver homogenates from chimpanzees with experimental NANB hepatitis. Susceptibility to NANB hepatitis of >150 chimpanzees in laboratories around the world has been >70%; among colony-born chimpanzees given pedigreed inocula, susceptibility has ranged between 95% and 100% (29). Successful infections have been achieved by intravenous, intramuscular, subcutaneous, and intrahepatic injections of inocula ranging in size from as little as 0.1 ml to as much as 3-75 ml, and one inoculum has been shown to have an infectivity titer in chimpanzees of ≥10⁸ infectious U/ml (24); however, severity of hepatitis in chimpanzes has not been related to inoculum volume or route of administration. Nor has there been a change in severity of NANB hepatitis after serial passage in chimpanzees (22,24). Incubation periods have tended to be the same for all animals receiving the same inoculum, but both short (2-6 wk) (3,5,6,10,22,25) and long (10-20 wk) (4,5,7,24) incubation periods have been observed. In some studies, the incubation period in the experimentally infected chimpanzee has paralleled closely the incubation period in the donor or patient source (6,10,22), while in other studies, the duration of the incubation period in experimental infection has not "breached the limits," i.e., has been longer or shorter than in its natural counterpart (3,4,7). In almost all cases, acute illness has been relatively mild, and peak alanine aminotransferase (ALT) levels have been substantially lower than in human NANB hepatitis, usually in the range of 100-300 IU, and rarely >400 IU (24,26). Jaundice was almost never observed, and 1 chimpanzee succumbed during his fourth bout of experimental viral hepatitis, the last two of which were caused by NANB agents (9). The characteristic biphasic or episodic pattern of aminotransferase activity elevation observed in human NANB hepatitis was seen also in experimentally infected chimpanzees (3-5,7,8,10), but the pattern of ALT elevation does not breed true during serial passage (22,29) and, therefore, cannot be used reliably to identify a specific inoculum, strain, or type of NANB agent as suggested by Shirachi et al. (30) and Tateda et al. (31).

Studies in chimpanzees have been useful in defining the duration of viremia during acute illness and in corroborating evidence of observations in humans that NANB hepatitis is associated with a chronic carrier state and with chronic hepatitis. Duration of viremia during acute NANB hepatitis has been documented by successful subpassage of illness to chimpanzees as early as 12 days before the onset of clinical illness (10) and as late as 10 wk after the onset of hepatitis (13 wk after inoculation, 1 wk after peak serum aminotransferase elevation in a chimpanzee whose illness began 3 wk after and ended 24 wk after inoculation) (32). Considered together with a volunteer study in which viremia was observed within a week of inoculation (2) and a natural outbreak in which viremia was shown to persist 34 days (32), these studies in chimpanzees confirm that viremia in acute NANB hepatitis can be quite prolonged. Moreover, like studies in volunteers (1), studies in chimpanzees have demonstrated that NANB hepatitis may cause chronic infection. Chronicity of infection has been confirmed by transmitting hepatitis to chimpanzees from donors and other chimpanzees implicated as long as 1-6.5 yr earlier in transmitting disease to intended or accidental recipients (3,4,33). Whereas some investigators have observed chronic hepatitis only rarely in experimentally infected chimpanzees (29), others have described chronic biochemical abnormalities for >1 yr in 40%-80% of experimentally infected chimpanzees; in some of these animals biochemical abnormalities have been observed for >3 yr (23,27).
Moreover, chronic viremia in chimpanzees has been documented by transmission of NANB hepatitis to recipient chimpanzees after inoculation with plasma obtained 8.5–21 mo after inoculation from donor chimpanzees with NANB hepatitis (23,27). Infection with NANB hepatitis has been transmitted to recipient animals with inocula derived from human and chimpanzee donors with chronic infection even when ALT levels in the donor had returned temporarily or permanently to normal (23,27). In chronically infected chimpanzees, even when ALT levels have returned to the normal range, morphologic lesions have been shown to persist (23,27). Episodes have also been described of recrudescence of clinically severe disease 2–3 yr after the onset of acute infection in experimentally infected chimpanzees (28). Generally, however, such chronic infections in chimpanzees tend to be asymptomatic as well as biochemically and morphologically mild.

Morphologic changes detectable by light microscopic evaluation of the liver in chimpanzees with NANB hepatitis reflect hepatocellular injury with greater sensitivity than biochemical changes. Except for a small proportion of animals in which the liver lesion resembles that seen in HBV-infected chimpanzees, in most chimpanzees with NANB hepatitis the liver is characterized by conspicuous variation in hepatocyte nuclei; diffuse, irregular clumping and vacuolization (steatosis) of the cytoplasm; prominent portal inflammation; rare foci of cytolytic necrosis; and conspicuous acidophilic bodies (34,35). A striking feature, as in humans, is the marked and enduring increase in and activation of sinusoidal lining cells, as well as the relatively subdued parenchymal lymphocytic reaction in these animals (34,35). In chimpanzees with chronic NANB hepatitis, similar changes occur, but less conspicuously so. Occasionally, rare erosion of the limiting plate and even central-portal bridges of necrosis and collapse can be seen (34). The chronic lesion, however, is relatively mild, resembles chronic persistent hepatitis (23,27), and neither chronic active hepatitis nor cirrhosis have been recorded (23,27,34,35). Although striking cytoplasmic and nuclear changes have been described in electron micrographs of liver biopsy specimens from chimpanzees with NANB hepatitis (see later discussion), no light-microscopic counterpart of these alterations has been observed, nor has the bile duct lesion of human NANB hepatitis appeared in chimpanzee tissue (34–36).

Among the most important exploitations of such an excellent animal model have been attempts to identify virus antigen–antibody systems, virus particles, and distinctive ultrastructural changes in the liver associated with NANB hepatitis. These will be described later. In addition, pedigreed inocula and susceptible chimpanzees have been used by several investigators to define such properties of these agents as their susceptibility to inactivation. Tabor et al. (37) have shown that formalin, 1:1000, at 37°C for 96 h, inactivates a transmissible strain of NANB hepatitis, and Yoshizawa et al. (38) inactivated one type of NANB hepatitis agent with formalin, 1:2000, at 37°C for 72 h or by heating at 100°C for 5 min. Such inactivation with formalin, even of undiluted material, suggests that formalin inactivation would be useful for production of an NANB hepatitis vaccine and that formalin treatment of hepatitis B vaccine is sufficient to inactivate any NANB virus(es) in the starting plasma pool from which this recently available vaccine is prepared. Preliminary studies suggest also that heating at 60°C for 10 h, the process used currently to treat heat-stable plasma derivatives, such as albumin and protein plasma fraction, is sufficient to inactivate NANB hepatitis agent(s) (28). Similarly, heating has been shown to inactivate an NANB hepatitis agent in antihemophilic factor VIII concentrates without reducing clotting factor potency (39). A combination of β-propiolactone and ultraviolet radiation treatment of source plasma has been used to inactivate hepatitis agents in factor IX concentrates, and material treated in this way did not transmit NANB hepatitis to chimpanzees (40); however, infectivity of the untreated inoculum and susceptibility of the recipient chimpanzees were not demonstrated. Recent studies indicate that the NANB agent most prevalent in American transfusion recipients is smaller than poliovirus (41,42) and that the “H” strain of NANB hepatitis (see later discussion) is inactivated by chloroform (43,44). In addition, preliminary findings suggest that at least one NANB inoculum in factor VIII is inactivated by chloroform, while another factor VIII-derived inoculum is resistant to inactivation by chloroform (Bradley DW, personal communication). Additional studies are underway to determine the physical properties of NANB agents and the susceptibility of these agents to other inactivation methods.

The chimpanzee model has also been instrumental in establishing beyond doubt that NANB hepatitis is not related to known human hepatitis agents. In none of the chimpanzees infected experimentally with NANB hepatitis agents did serologic evidence of infection with HBV, HAV, cytomegalovirus (CMV), or Epstein–Barr virus (EBV) develop, and no such serologic markers have become expressed during serial passage of NANB hepatitis in chimpanzees. Moreover, chimpanzees immune to HAV and HBV readily became infected with NANB hepatitis, and chimpanzees infected with and immune to NANB hepatitis were easily infected later with HAV and HBV (21). In short, there has been no demonstra-
ble heterologous immunity between NANB hepatitis and other hepatitis agents. Similarly, chimpanzees, readily infected with NANB hepatitis, are not susceptible to infection with the GB hepatitis agent transmitted in marmosets and identified originally by Delhard et al. (45), i.e., GB is not the NANB agent being seen so frequently in post-transfusion hepatitis (46). An interesting observation in this vein was the demonstration that in chimpanzees with chronic HBV infection, superinfection with NANB agents led to a transient suppression of the expression of HBV markers in the liver and serum (47).

Availability of an animal model provides a method to compare immunologic relationships among different inocula and to determine whether different NANB agents exist, as suggested by epidemiologic data (48–50) and ultrastructural findings (51). Tabor et al. (52) found that one NANB inoculum conferred immunity against two others from geographically diverse regions, and cross-challenge studies with two different inocula studied at the National Institutes of Health (NIH) failed to support any convincing immunologic distinction between them (24, 25, 29). In contrast, other cross-challenge experiments in chimpanzees suggest that there are different NANB agents. Tsiquaye and Zuckerman (9, 53) demonstrated that the NANB agent in a factor IX concentrate (7) did not confer immunity in a chimpanzee against a second NANB agent in a factor VIII concentrate (8); therefore, the two agents are immunologically distinct. Bradley et al. (20) confirmed the distinction between these factor VIII and factor IX inocula, but demonstrated that the factor VIII inoculum is immunologically similar to the “H” inoculum of the National Institutes of Health (NIH) (see later discussion) (24, 25, 51). Similarly, cross-challenge experiments by Yoshizawa et al. (54) are consistent with the existence of two different NANB agents, each associated with unique viruslike particles and ultrastructural changes (see later discussion). Hollinger et al. (19) also demonstrated the presence of two different NANB agents, one in factor VIII, by successful reinfection of chimpanzees with a second inoculum 2 yr after infection with an original inoculum. Tabor et al. (29) recently identified a NANB agent in a fibrinogen preparation that is distinct from the NANB agent they found so geographically prevalent in the United States (52). Although interpretation of these studies may be complicated by the chronic relapsing pattern of ALT activity after a single NANB infection, and although one group of investigators has observed absence of homologous immunity in chimpanzees after NANB hepatitis infection (Prince AM, personal communication), these studies, taken together, still strongly suggest that there are at least two different NANB hepatitis viruses.

Marmosets

In addition to chimpanzees, marmosets have been studied as a potential animal model for NANB hepatitis. Although Tabor et al. (55) found no indication of susceptibility to NANB hepatitis in 6 marmosets (Saguinus mystax and S. labiatus) followed for 3 mo after inoculation, Feinestone et al. (24, 25) have demonstrated that these species of marmosets are susceptible and that NANB hepatitis agents can be passaged serially in marmosets. Although the infectivity titer of one inoculum was higher in marmosets than in chimpanzees, marmosets were not as readily or reproducibly infected. A lower proportion were successfully infected, and the incubation period was long, 13–24 wk in 80% of infected animals, i.e., longer than the observation period in the studies of marmoset susceptibility by Tabor et al. (55). Cross-challenge studies in marmosets between NANB hepatitis agents and the GB hepatitis agent confirm the immunologic distinction between these two agents, and differences in histologic changes in the livers of marmosets between animals with NANB and GB hepatitis have also been identified (25, 26). Successful infection of marmosets has also been described by Fields et al. (57). Although serial passage of NANB hepatitis agents in marmosets may improve the susceptibility and reliability of this experimental animal model, currently observed variability in resistance and susceptibility limits its usefulness. Attempts to infect a large variety of cell lines (41) and athymic nude mice with NANB hepatitis have been unsuccessful (58), and no other experimental hosts for these agents have been suggested. Discovery of the chimpanzee (and marmoset) model is an important breakthrough, however, and, undoubtedly, will contribute to the quest for a specific immunologic test to identify NANB hepatitis infection and infectious blood donors.

Putative Non-A, Non-B Agents and Immunologic Markers

Approaches used in the past to identify hepatitis A and B antigens and virus particles have been applied to the search for NANB antigen(s) and virus particles, and the discovery of an animal model has accelerated this area of investigation. In fact, the number of reports in which putative NANB antigen–antibody systems and virus particles have been described has increased rapidly during the last few years, and an uninitiated reader might conclude from this burgeoning literature, as some have (59).
that a breakthrough had been achieved. Unfortunately, to date, NANB hepatitis agents have not been cultured in vitro, and, despite a plethora of reports to the contrary, no viral agent or immunologic marker has been identified that fulfills accepted serologic criteria for a specific association with NANB hepatitis. On the other hand, although routine availability of specific serologic markers for the diagnosis and prevention of NANB hepatitis remains an elusive goal, promising leads are certain to arise from the preliminary studies already reported.

Immunodiffusion

One of the first approaches to be apparently successful was agar gel immunodiffusion. Using convalescent serum as antibody, Shirachi et al. (30) detected a “hepatitis C” antigen in acute-phase serum samples from 17 of 23 patients with posttransfusion NANB hepatitis. This antigen had a peak buoyant density in cesium chloride of 1.35–1.38 g/ml, at which densities viruseslike particles of various diameters (30 nm, 40 nm, and 60 nm) were observed (60), migrated as a β-globulin, and had a molecular weight of ~100,000–300,000 daltons (30,60). In the initial report, this antigen was universally present in long-incubation and less commonly observed in short-incubation NANB hepatitis. Antibodies to the antigen were rarely detected, and then, only transiently. Questions about the specificity of this antigen—antibody system were raised, however, by the detection of the antigen in pretransfusion serum from several patients with transfusion-associated hepatitis and in serum from 2 of 9 patients with acute hepatitis B (30). In subsequent studies, these investigators failed to identify many more patients with “hepatitis C” antigen, could not identify virus particles in the immunoprecipitates, and found that, not infrequently, a serum sample that contained a putative antibody when tested against an earlier serum contained antigen activity when tested against a later serum (61).

Still, this initial report was followed by many others in which putative NANB antigen—antibody systems were identified by immunodiffusion. Vitvitski et al. (62) described an immunodiffusion test for NANB antigen and antibody in which the sources of antibody consisted of convalescent serum from a patient with NANB hepatitis, serum from a homologous that had received multiple transfusions, and serum from a chimpanzee hyperimmunized after recovery from experimental NANB hepatitis with liver homog enate from another chimpanzee with acute NANB hepatitis. Two parallel antigen—antibody precipitin lines were detected between those antibody reagents and acute-phase serum from 9.5% of sporadic, 17% of retrospectively evaluated posttransfusion, and 86% of prospectively evaluated posttransfusion NANB hepatitis cases, but not in pretransfusion serum or serum from patients with hepatitis A or B, with drug-induced hepatitis, or in normal bleed donors’ serum. In addition, identical antigens to those in the serum of NANB hepatitis patients were detected by immunodiffusion in liver homogenates. Antibody to this antigen was detected during convalescence in 36% of cases, and globulin from such serum, labeled with fluorescein, was used to stain NANB antigen in nuclei of hepatocytes from patients with acute NANB hepatitis, but not in normal control tissue. Comparison of this antigen with that of Shirachi et al. (30) showed that there was immunologic identity between Shirachi’s “hepatitis C” antigen and one of the two parallel “NANB antigen” precipitin lines of Vitvitski et al. (62).

Further characterization of this antigen—antibody system by Vitvitski, her coworkers Trepo and Prince, and other colleagues (63–69) showed that it cross-reacted with one specificity of hepatitis B e antigen—HBcAg/3. Furthermore, these investigators described an antigen—antibody system analogous to and crossreacting with the hepatitis B core antigen (HBcAg)/anti-HBc system of HBV. The HBcAg-like antigen could be detected in liver cell nuclei, and, simultaneously, antibody to this antigen could be detected by indirect immunofluorescence or counterimmunoelectrophoresis in the serum of patients with NANB hepatitis (68,66,67,68,70–73). Patients with this NANB core antigen in the liver and antibody to this antigen in serum have also been found to have 22–25 nm viruslike intranuclear inclusions, reminiscent of HBV cores, in their hepatocytes (68,71,74). In addition, these investigators identified yet another NANB antigen by immunodiffusion which, they claim, is analogous to but does not crossreact with the surface antigen of HBV, and may even be expressed on the surface of 37-nm Dane-like particles they have identified in the serum of patients with NANB hepatitis (68,66,67,68,70–73). Patients these investigators have observed a variety of particles ranging in size from 4 nm to 37 nm (most of which resemble lipoproteins), in several samples, morphologic forms resembling HBV were detected, including, in addition to the 37-nm Dane-like double-shelled particles, 15–25-nm spherical and tubular particles. Moreover, the 37-nm particle, it was claimed, contained deoxyribonucleic acid (DNA) and an endogenous DNA polymerase. The DNA polymerase could be detected in the serum of patients with NANB hepatitis. These investigators, therefore, concluded that NANB hepatitis virus is similar in its properties to HBV and other HEPADNA viruses and should be classified with them and that
the three antigens should be designated "NANBsgAg", "NANBcAg", and "NANBeAg." In
>90% of NANB cases and in a substantial proportion of
patients with HBsAg-negative chronic hepatitis
and hepatitis in dialysis units, one or more of these
antigens, antibodies, or both, could be detected (63–
81). Further support for their hypothesis that NANB
hepatitis has properties like HBV, but is immunolog-
ically distinct, came from studies in chimpanzees
vaccinated against and immune to HBV infection.
When inoculated with an NANB hepatitis inoculum
containing these NANB antigens, these nonhuman
primates became infected with NANB hepatitis and
expressed the same NANB hepatitis antigens in
serum and liver (15,16).

Although an attractive hypothesis and encourag-
ing findings, corroboration by other investigators has
not been forthcoming. Prince (82,83) reported subse-
quent that difficulties with the specificity and
reproducibility of this system were encountered and
that he could not confirm the original findings,
especially in experimentally infected chimpanzees.
Even Trepo and colleagues have reported that Dane-
like particles are rarely detected in NANB hepatitis
sera (68), that "unexplained results" (68) occur occa-
sonally in their studies designed to test the validity
of these antigen–antibody systems, that chimpanzee
sera cannot be analyzed with any confidence be-
cause too many "nonspecific antihuman serum reac-
tions" appear in the immunodiffusion test system
(68), that many nonspecific immunodiffusion lines
appear in NANB hepatitis sera that often cannot be
readily distinguished from the specific NANB pre-
cipitin lines (68), and that serologic responses ap-
ppear in some patients before transfusion (84). Dane-
like particles have been observed by only one other
investigator (85), but not by any of the many others
who have studied NANB hepatitis, and the serologic
relationship of these particles to NANB hepatitis has
not been demonstrable. At least one of the antigens
defined by immunodiffusion can be no more specific
than that described by Shirachi et al. (30), with
which it is identical. Adding further to the confu-
sion, Ishida et al. (61) have reported that the "hepa-
titis C" antigen of Shirachi et al. (30) bears no relation-
ship to any specificity of HBsAg (or HBeAg).

Furthermore, an immunologic association between
HBV and NANB hepatitis has not been observed by
other investigators (see later discussion), and chim-
panzees infected experimentally with NANB hepato-
tis, including those studied by Prince (63), do not
express any HBV markers, including HBeAg, wheth-
er following primary inoculation or serial subpas-
sages (3,22,29).

Other investigators have described one or more
NANB hepatitis antigen–antibody systems by
immunodiffusion (66–101), and exchanges of re-
agents among the laboratories in which immunodif-
fusion antigen–antibody systems were developed
have shown that many are identical (30,62,95,99).
Suh et al. (102) identified an antigen–antibody sys-
tem by immunodiffusion that was shown to be
identical with others that had been compared with
and found to be immunologically indistinguishable
from those of Shirachi et al. (30) and the NANBeAg
of Vititski, Trepo, and colleagues (82). In contrast to
the specificity of this immunodiffusion system re-
ported by others, Suh et al. (102) reported findings
that challenged its specificity. These investigators
detected the antigen in a high percentage of patients
with other types of viral hepatitis and nonviral liver
diseases (such as hemochromatosis and primary
biliary cirrhosis) and in patients with systemic lupus
erythematosus and rheumatoid arthritis, which, like
NANB hepatitis (103), are associated with high lev-
els of circulating immune complexes. Antibody
defined by this system was found to be widely
distributed in normal blood donors. Even more dis-
concerting than the nonspecificity of the system was
the finding that the apparent antibody did not have
the biochemical properties of an immunoglobulin,
and that the apparent antigen had the immuno-
chemical properties of immune complexes. Similar
findings were reported by Hoofnagle (87), who showed
that the precipitin system in NANB hepatitis occurs
in patients with other types of acute and chronic
liver diseases and that the constituents of the
immuno-diffusion system do not have the properties
of an antigen–antibody precipitate. An additional
note of confusion was added by the work of Hopkins
et al. (95,104,105), who described an antigen–anti-
body system identified by immunodiffusion. Like
Suh et al. (102), Hopkins et al. exchanged reagents
with other investigators and found that their system
was immunologically identical to Shirachi's "hepa-
titis C" or Trepo's "NANBeAg" systems; however,
the antigen in the system of Hopkins et al. (104) was
identical to the antibody in the system of Suh et al.
(102) and vice versa. Edelson et al. (106) pointed to
this unusual reversibility of purported immunologic
reactants as evidence that neither of the two systems
has the properties of an immunoprecipitate. As
Hoofnagle and Feinstone (107) have pointed out, the
likelihood is remote that such elusive virus antigens
and antibodies as those associated with NANB hepato-
tis could be identified by immunodiffusion, which
has such a high sensitivity threshold, ~0.5 μg/ml of
protein.

The putative NANB hepatitis antigens defined by
immunodiffusion have never been shown convinc-
ingly, either retrospectively or prospectively, to
identify units of blood implicated in the transmis-

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sion of NANN hepatitis. The most deprectrating evi-
dence against the validity of the NANN antigen-
abody systems identified by immunoaifusion
comes from the poor performance of such assays on
coded panels of serum samples. Two such panels,
consisting of samples from pedigree posttransfu-
sion NANN hepatitis cases and implicated blood
donors (some of which had been proven infectious
in chimpanzees), liver disease controls, and pedi-
greed normal blood donors, were assembled and
distributed by Alter and colleagues (44,108) before
International meetings in June of 1980 and March of
1981. Participating laboratories, including at least
seven of those reporting the major immunodiffusion-
tests described earlier, failed to break the code suc-
cessfully on either of the two panels. The immuno-
diffusion tests were found to be not only insensitive
and nonspecific, but also poorly reproducible: con-
cordance was not infrequently lacking between du-
plicate samples of the same serum tested separately
under code by the same laboratory. Not unlikely,
these precipitates in immunodiffusion gels represent
nonspecific acute-phase reactants, circulating im-
mune complexes, Immunologic responses to liver or
other serum- or tissue proteins—such as autoantibio-
dies and antibodies to albumin, both of which are
prevalent in NANN hepatitis (100,109). One group
has shown that a test for a putative NANN hepatitis
antigen they had described was actually detecting an
IgM anti-IgG rheumatoid factor (RF) that could not be
demonstrated by conventional RF assays (110). In
fact, many of the tests that performed poorly on
coded panels of sera did positively identify an
NANN hepatitis serum that contained RF (44). Anti-
body responses to a variety of nonspecific bacterio-
logic, virologic, onctic, and self-proteins and circu-
lating immune complexes (111–115) have been
observed in patients with acute and chronic hep-
itis and appear to reflect a transient loss of liver
reticuloendothelial cell modulation of immunologic
responsiveness. The occurrence of these nonspecific
immunologic responses provided a pitfall for some
investigators in the interpretation of serologic tests
for hepatitis A almost a decade ago (110). Apparent-
ly, these same difficulties are confounding inter-
pretation of serologic responses in patients with NANN
hepatitis.

Counterelectrophoresis

Using reagents from experimentally infected
chimpanzees, Tabor and Gerety (117) described a
counterelectrophoresis (CEP) test for a putative
NANN hepatitis antigen and antibody. The soluble
antigen, apparently indistinguishable immunologi-
cally from that detected with immunodiffusion by
Shirachi et al. (30), was detected intermittently in
serum samples from 6 of 7 chimpanzees with experi-
mental NANN hepatitis, predominantly during peri-
ods of elevated serum aminotransferase activity.
Convalescent serum from these chimpanzees was
used as a source of antibody. Antigen was undetect-
able in preinoculation serum of chimpanzees with
NANN hepatitis or in serum obtained during acute
illness from chimpanzees with hepatitis A or B, but
was detected in serum from several humans with
chronic NANN hepatitis whose blood had transmit-
ted the illness to other humans and chimpanzees, as
well as in the serum of 11 of 31 blood donors whose
blood had been implicated 1–5 yr previously in
cases of transfusion-associated hepatitis. Moreover,
the antigen persisted for >6 yr in the circulation of
an asymptomatic person whose serum remained
infectious and continued to transmit NANN hepatitis
to experimentally inoculated chimpanzees during
that period (33). Antibody was detected in convales-
cent serum from 7 of 7 chimpanzees, and in con-
valence serum from 2 humans with NANN hepatitis,
and as well as in 5 of 20 blood donors implicated in the
past as having transmitted hepatitis to blood recipi-
ents. Despite having been patented in the United
States, however, this antigen—antibody system was
beset with difficulties related to specificity when
applied to human samples, and, identical with that
described by Shirachi et al. (30), lacks strict specific-
ity for NANN hepatitis. Another problem that con-
fronted interpretability and raised questions about
specificity of this test was the fact that antisera
used in the CEP assay also detected a normal liver
antigen (117). Finally, CEP assays have not per-
formed well on the coded serum panels with which
the validity of the immunodiffusion tests were chal-
lenged; both false-positivity and nonreproducibility
were encountered (44,108). Similar CEP antigen-
abody systems have been reported by several
other investigators (92,118,119) but have not been
described in detail or subjected to validation.

Immunofluorescence

Several groups have applied immunofluores-
cence techniques to the quest for NANN antigens and
have described the presence of NANN virus antigens
in liver biopsy specimens of chimpanzees or humans
with NANN hepatitis (14,62,68,101,120–125). Gerety
and Tabor (121) and colleagues used convalescent
serum from experimentally infected chimpanzees to
identify an intrahepatic NANN antigen in chimpan-
ze liver biopsy specimens. Although in a prelimi-
nary report Tabor described immunofluorescence
staining in hepatocyte cytoplasm, associated with the
presence of cytoplasmic 20–22-nm particles ob-
served by electron microscopy (126), Kabiri, Tabor, and Gerety (122), in a more definitive report, demonstrated nuclear staining by indirect immunofluorescence in chimpanzee liver biopsy specimens incubated with convalescent serum from 1 of 10 infected chimpanzees and from a patient with NANB hepatitis. Nuclear antigen became detectable when serum aminotransferase levels rose and persisted in many cases even after these enzyme levels returned to normal. Except for 1 chimpanzee that had nuclear antigen in the liver before inoculation (postulated to have been chronically infected before experimental inoculation), none of the other 9 chimpanzees with NANB hepatitis had intranuclear antigen or circulating antibodies, detected by indirect immunofluorescence against antigen-positive liver specimens, before inoculation. Similarly, no immunofluorescence staining was detected in liver biopsy specimens from chimpanzees with experimental hepatitis A or B or in uninoculated controls or when convalescent serum from hepatitis A or B was used as the antibody probe for nuclear antigen. Furthermore, the antibody to intrahepatic nuclear NANB antigen was shown to be unrelated to anti-DNA, RF, antinuclear antibody, or antibody to the HBV-associated delta agent (122). Specificity of this chimpanzee antigen–antibody system, however, has been less convincing in material obtained from human sources.

A nuclear antigen was detected by immunofluorescence in the livers of patients with acute and chronic NANB hepatitis by Vilyviski and Trepo (62) and colleagues, as noted earlier, and the antigen–antibody system defined in this way was felt to resemble the core antigen–antibody system of HBV (67,68,71,72). A similar nuclear antigen and circulating antibody were identified by Alberti et al. (123), and by exchanging reagents, Trepo et al. (68) found a high degree of concordance between the immunofluorescence systems described in these two laboratories. In addition, nuclear staining distinct from HBCAg was detected by immunofluorescence by Villa et al. (120) in liver biopsy specimens from 3 patients with non-B chronic active hepatitis. Arnold et al. (cited in 101) and Do Vos et al. (14) have also detected a nuclear NANB antigen in human (14,101) and chimpanzee (14) liver by immunofluorescence.

Although the independent description of relatively similar antigen–antibody systems identified by immunofluorescence seemed promising, these antigen–antibody systems, like the others described above, have proven to be flawed. In some of these studies, demonstration of the specificity of immunofluorescence staining with the appropriate blocking procedures was not done (14,122,123). In others (62,72,120), blocking of staining was demonstrated with unlabeled antisemur or by incubation with crude homogenates of liver from patients with NANB hepatitis. As Hoofnagle and Feinestone (107) have pointed out, however, more appropriate blocking studies, with purified antigen and antibody reagents from NANB hepatitis cases other than those being studied and with comparably prepared control human liver-derived antigen and serum antibody, were not done. Analysis of these systems reveals that the antibodies were often detectable at very low, unconvincing titers and that antigen (122) or antibody (123) were detectable in a proportion of control samples. Moreover, others have failed to reproduce the apparent specificity of these findings. Hoofnagle and Feinestone (107) found that nuclear antigen could be detected readily by indirect immunofluorescence staining with antibody derived from convalescent serum in liver biopsy specimens from humans and experimental animals with NANB hepatitis. Such staining, quite comparable to that reported by others, however, was found to be nonspecific for NANB hepatitis, and similar nuclear staining was observed in all liver samples, including preinoculation liver from NANB hepatitis cases and liver biopsy specimens from hepatitis A and B cases. Finally, putative immunofluorescence tests for NANB corelike antibody have been subjected to validity testing by assessing their performance in discriminating between NANB hepatitis cases and controls in the coded serum panels of Alter et al. (108). An indirect immunofluorescence test for this antibody was found to have poor reproducibility (only 77% concordance between duplicates of the same serum sample), to be insensitive (positive in none of 11 cases of NANB hepatitis), and to be nonspecific (positive in 1 of 2 disease control samples) (108).

Electron Microscopy

Another tool that has been applied to the search for NANB agents is electron microscopy. Bradley et al. (8) identified 27-nm viruslike particles, morphologically indistinguishable from HAV particles, in the liver homogenate from a chimpanzee with acute NANB hepatitis after inoculation with a lot of clotting factor VIII concentrate that had been implicated in the transmission of NANB hepatitis to humans. To demonstrate these particles, Bradley et al. (8) used the technique of immune electron microscopy and incubated chimpanzee liver homogenate fractions, factor VIII, or acute-phase serum with convalescent serum from a patient with factor VIII-induced NANB hepatitis. Apparently-antibody-coated aggregates of relatively amorphous particles, with a buoyant density of 1.31 g/mm, were observed also
in gradient fractions of the factor VIII inoculum, in acute-phase serum from 1 of the patients with factor VIII-associated acute NANB hepatitis, as well as in acute-phase serum from 2 unrelated cases of NANB hepatitis, and in liver homogenates from chimpanzees infected in serial subpassage with particle-containing liver homogenate fractions from other experimentally infected chimpanzees (6). Although these particles were identified in livers of other chimpanzees in the serial subpassage chain, subsequent attempts to recover viruslike particles from the original liver in which they were found were not rewarding (6). Most importantly, these particles were aggregated as well in the absence of convalescent antibody, invalidating any confirmation of a serologic relationship between the particles and NANB hepatitis. Morphologically similar 27-nm viruslike particles with a density of 1.28 g/ml were described by Yoshizawa et al. (6,54) who used convalescent serum from a chimpanzee with experimental NANB hepatitis as an antibody reagent for immune electron microscopy. The chimpanzee had been inoculated with a fibrinogen preparation that had been implicated in transmitting NANB hepatitis to 2 patients. With this immune electron microscopic technique, applied under code, Yoshizawa et al. (6) detected particles in the fibrinogen preparation and in serum of 8 of 100 apparently healthy blood donors with elevated serum ALT levels, one of which was shown to transmit NANB hepatitis to 2 chimpanzees (in both of which circulating 27-nm particles could be demonstrated). Specificity of the reaction between convalescent serum and the virus particles was shown by the failure of chimpanzee preinoculation serum or sera with antibodies to HAV and HBV antigens to aggregate the particles; however, other controls, such as nontreated fibrinogen, preinoculation and convalescent serum from chimpanzees with type A and type B hepatitis, and serum from pedigreed normal blood donors whose blood had been found free of NANB agents, were not described. Furthermore, the published photographs of the apparent immune electron microscopic aggregates resemble, and may represent, nonspecifically agglutinated particles (6). Two other viruslike particles have been identified by immune electron microscopy in NANB hepatitis sera. Marciano-Cabral et al. (119) detected 22-nm particles in serum of 3 patients with chronic NANB hepatitis whose sera were incubated with convalescent serum. A second viruslike particle, 25 nm in diameter, was identified with immune electron microscopy by Yoshizawa et al. (54) in the serum of an apparently healthy blood donor. As mentioned earlier, the 27-nm-particle-containing inoculum (NANB-1) and the 25-nm-particle-containing inoculum (NANB-2) were shown to be different by chimpanzee crossinoculation studies (54). Neither the 22-nm particle of Marciano-Cabral et al. (119), nor the 25-nm particle of Yoshizawa et al. (54) has been tested adequately for a serologic relationship with NANB hepatitis.

Besides these viruslike particles resembling HAV (6,8) and those described previously resembling HBV (75,76), a variety of other viruslike particles have been described in materials from patients and experimental animals with acute and chronic NANB hepatitis and implicated donors or in blood products. These include 60–80-nm particles resembling C-type ribonucleic acid (RNA) viruses in chimpanzee liver (126); 30-nm, 40-nm, and 60-nm particles in human serum (60,81); 20–22-nm cytoplasmic particles in chimpanzee liver (126); paroviruslike 23-nm particles in human serum (127); 38–44-nm papovavirus particles in urine (128); 80–140-nm paramyxovirus particles in serum (88,89); 27–29-nm particles in the stools of a patient with the water-borne type of NANB hepatitis in India (Sreenivasan MA, Sehgal A, Prasad SR, Dhorje S, Pavri KM, personal communication, 1982); 32-nm enveloped, hexagonal particles with 22-nm inner cores, as well as freely circulating cores in human serum (129,130); 18-nm and 37-nm particles in serum, as well as 14–37-nm particles in liver specimens evaluated by thin-section electron microscopy (119); particles 60 nm in diameter with a 40-nm core in urine (131); 36-nm and 61-nm particles in plasma (132); 29–34-nm particles in liver homogenates (97); not to mention the large number of virus particles isolated in the past from patients with hepatitis (133). In addition, 20–22-nm viruslike particles have been detected in serum (134) and 34–36-nm particles in liver homogenates (135) of marmosets infected with the GB hepatitis agent, the original hepatitis agent apparently transmitted from human to marmoset, which is distinct from human HAV, HBV, and the most prevalent form of NANB hepatitis (46), but still a NANB candidate. No serologic relationship between these particles and NANB hepatitis, however, has been demonstrated, and the multiplicity of virus types and sizes observed underscores the caution with which those reports must be interpreted. Those who have extensive experience with electron microscopy of human tissues and fluids are well aware of the ubiquity of visual artifacts and viruslike particles in those materials.

**Ultrastructural Alterations**

A number of changes have been observed by electron microscopy in the livers of experimental animals and humans with NANB hepatitis. Shimizu et al. (51) detected two different types of ultrastructural changes in liver biopsy specimens from chim-
panzees with experimental NANB hepatitis. All chimpanzees infected with one inoculum (strain F), after which hepatitis had developed with a mean incubation period of 11 wk, had double-membraned tubular cytoplasmic structures in the cisternae of dilated rough endoplasmic reticulum during the time of elevated serum aminotransferase activity (but not before or after) (Figure 1). Similar “undulating tubules” were described in the livers of chimpanzees with acute NANB hepatitis by Jackson et al. (136). In these animals, hepatocyte nuclei were normal. In contrast, all infected chimpanzees that had been given a different inoculum (strain H) and in which hepatitis developed 6–7 wk after inoculation had no cytoplasmic inclusions. Instead, nuclear changes were observed during the period of elevated aminotransferase activity (but, again, not before or after) (51). Hepatocyte nuclei appeared to be heterogeneous in density, condensed, and irregularly shaped, and contained aggregates of 20–27-nm particles, not sufficiently uniform to resemble viruses (Figure 1). Under code, Shimizu et al. (51) could discriminate between chimpanzees inoculated with strain F and strain H by detecting the appropriate ultrastructural change; neither of these alterations were detected in livers from chimpanzees with hepatitis types A or B. In all liver biopsy specimens from chimpanzees with experimental NANB hepatitis, neither the cytoplasmic or the nuclear changes, never both, were observed by electron microscopy. The apparent virus specificity of these ultrastructural alterations was confirmed by Tsuquyo et al. (53), who also detected cytoplasmic changes exclusively in chimpanzees with long-incubation NANB hepatitis and nuclear changes only in chimpanzees with short-incubation NANB hepatitis. Identical or similar intranuclear and cytoplasmic ultrastructural changes have been observed in liver biopsy specimens of both chimpanzees and humans with acute and chronic NANB hepatitis by other investigators (13, 14, 74, 119, 137–152); however, the hypothesis that these changes define two distinct NANB inco-

Figure 1. Electron micrographs of hepatocytes in liver biopsy specimens from chimpanzees with experimental NANB hepatitis. A. This chimpanzee was infected with the strain “H” inoculum. The hepatocyte nucleus is contracted, the chromatin is clumped, and there is a cluster of irregular nuclear particles (arrow and inset). B. This chimpanzee was infected with the strain “F” inoculum. The characteristic double-membrane tubular structures are prominent in the cytoplasm. The bar represents 1 μm (100 nm in inset). These photomicrographs were kindly provided by Stephen A. Feinstone, M.D., and are reproduced with his permission and that of The University of Chicago Press from The Journal of Infectious Diseases 1981;144:508–98 (24).
and nuclear changes can occur in the same liver biopsy specimen (119,141,142,144) or sequentially (140) in the same animal. Although one inoculum (the F strain) has been associated only with cytoplasmic changes, the inoculum associated originally with nuclear changes (the H strain) has now also been shown to provoke cytoplasmic alterations (24,25,141). Moreover, cross-challenge studies in chimpanzees between the F and H inocula failed to identify an immunologic distinction between them (24,25). Additional studies in chimpanzees have shown that the cytoplasmic changes do not necessarily occur in temporal association with changes of acute hepatitis as originally described; cytoplasmic alterations have been observed at variable times after inoculation, occasionally in early or pre-acute-phase liver biopsy specimens (153). Although these ultrastructural alterations have not been found in livers of humans or experimental animals with hepatitis types A and B, and, therefore, serve as useful markers of NANNB hepatitis, there is no evidence that they represent virus-related or virus-specific structures. Similar nuclear changes have been observed in human fibrous histiocytoma (25) and biliary atresia (154), and similar cytoplasmic changes have been described in hepatocellular carcinoma (149), early differentiating fetal lung cells, systemic lupus erythematosus, scleroderma, and polymyositis, among other disease states (140). These changes may represent a common metabolic response to certain types of tissue injury. Similarly, crystalline arrays in the cytoplasm of sinusoidal endothelial cells and Kupffer cells have been identified in chimpanzees with experimental NANNB hepatitis (20,148,155–157), however, similar changes have been observed in chimpanzees with experimental HAV infection (20), and additional analysis has revealed that these arrays lack the properties of viruses (157). Instead, they may represent a host cell response to infection (157). In any event, because detection of ultrastructural changes requires electron microscopic examination of liver tissue obtained during acute or chronic illness, these markers cannot be used in practical assays for NANNB hepatitis agents.

**Radioimmunoassay and Enzyme Immunoassay**

The most sensitive in vitro immunologic techniques applied to viral diagnostics are radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA). Both of these methods have been applied to detection of NANNB serologic markers. In 1978, Prince et al. (5) described a radioimmunoassay for "hepatitis C" antigen and antibody. The antigen was derived from liver tissue obtained from an experimentally infected chimpanzee with acute NANNB hepatitis and the antiserum from a multiply-transfused hemophilic. Counts per minute for positive specimens, however, were barely elevated, confirmatory neutralization tests were inconsistent, and interfering immunologic reactions were encountered between normal chimpanzee liver antigens and hemophilic serum. In short, subsequent investigation failed to uphold the reliability, reproducibility, or specificity of the test for NANNB hepatitis. A similar radioimmunoassay approach was described by Purcell et al. (158), who used acute-phase serum from patients with posttransfusion NANNB hepatitis as an antigen source and convalescent serum obtained from similar patients as well as serum from multiply-transfused patients as antibody probes. Apparently NANNB antigen activity was detected in two acute-phase serum samples; however, specificity of these antigen–antibody systems for NANNB hepatitis could not be demonstrated. In none of the patients studied, including the 2 with antigen in acute-phase serum, did antibody seroconversion occur, and in two-thirds of the patients with NANNB hepatitis, antibodies to these antigens were present in pretransfusion serum. Another radioimmunoassay was developed by Neurath et al. (159), who used IgG purified from a multiply-transfused person to detect an antigen, designated "hepatitis related antigen" (HRA), in a variety of patient groups. Among 15 cases of posttransfusion hepatitis, 100% had HRA in serum, and almost 80% of 21 hospitalization patients with NANNB hepatitis also had detectable circulating HRA. This antigen was also detected in 30% of hemophiliacs and in HBsAg-negative blood donors with normal (13%) HRA-positive or elevated (34% HRA-positive) ALT levels. In addition, HRA was shown to bear no immunologic or biochemical relationship to liver-specific proteins, α-fetoprotein, β-macroglubulin, carcinoembryonic antigen, liver-derived F antigen, or HLA specificities (159). Despite these promising findings, however, HRA was also detected in 69% of HBsAg-positive homosexual men and in 59% of Taiwanese blood donors with high serum HBsAg titers, in 3 of 5 homosexual men with acute HAV infection during the period of ALT elevation, and in the cytoplasm of several human embryonic and carcinoma tissue culture cell lines (159,160). These findings suggest that HRA is not specific for NANNB hepatitis but represents either a cytoplasmic alloantigen or a host antigen with monoclonal features that may be synthesized and released into serum in response to NANNB hepatitis, other hepatitides, and other infections (159,160).

More recently, RIA and ELISA tests have been described in preliminary communications by several investigators (16,161–166). Arnold et al. (161) used
convalescent serum as a source of antibody and detected an NAB antigen in 13 of 26 (50%) patients with acute NAB hepatitis, none of 10 patients in the late convalescent phase of NAB hepatitis, 14 of 16 (88%) patients with chronic NAB hepatitis after a plasmapheresis-associated outbreak, 11 of 20 (55%) patients with sporadic, HBsAg-negative, autoimmune marker-negative chronic hepatitis, but not in patients with HBsAg-positive chronic active hepatitis or healthy blood donors. No other controls were described in the original communication. Khan, Hollinger and their colleagues (164) used chimpanzee liver homogenate fractions as an antigen source and convalescent chimpanzee sera as antibody probes in an RIA and RIA-blocking test for NAB antigen and antibody. Antibody was detected primarily in the microsomal fraction containing endoplasmic reticulum, and antibody was detected in convalescent sera from chimpanzees with NAB but not type A or type B hepatitis. No other details of this assay have appeared. Duermeyer and Stue (18,162,163) have described an EIA in which convalescent NAB hepatitis serum was used as an antibody probe to detect a putative NAB antigen (designated DS antigen) in the serum of a hemophiliac. This antigen was detected in serum of 11 of 17 patients with posttransfusion NAB hepatitis and in serum of chimpanzees with factor VIII-induced NAB hepatitis, but not in serum of chimpanzees with factor IX-induced NAB hepatitis or controls with other liver diseases. These investigators concluded that this antigen was associated with the short-incubation type of NAB hepatitis seen after factor VIII infusion. A similar antigen–antibody system was identified by Luo, Thomas, and their colleagues (165,166) with an RIA in which hemophilic serum was used as the source of antibody to detect NAB antigen in serum. The antigen was detectable in serum of 9 of 18 (50%) hemophilic patients with acute, short-incubation, factor VIII-associated NAB hepatitis and 10 of 49 (20%) hemophilic patients with chronic NAB hepatitis. In this RIA, rheumatoid factor in serum occasionally gave rise to false-positive results, and several patients with other liver diseases (acute hepatitis B, HBsAg-positive chronic liver disease) were positive for this antigen in the absence of serum rheumatoid factor. Although this assay appears to be disease-associated with the short-incubation type of NAB hepatitis after factor VIII therapy, its performance in identifying the more commonly encountered, long-incubation posttransfusion NAB hepatitis remains to be evaluated.

These preliminary reports appeared promising and the RIA of Arnold et al. (161) performed very well on the coded serum panel of Alter et al. (108). Concordance between duplicates was 100% sensitivity was ~63%, specificity was 100% (no false-positive results), and the overall rate of correct responses was 78%. In addition, with this RIA, Arnold was able to detect antigen under code in a limited number of acute- and post-acute-phase serum specimens but not in preinoculation sera from chimpanzees with experimental NAB hepatitis (25). To date, however, despite this excellent performance on coded panels, >2 yr have elapsed since the preliminary description of this RIA (161) and its application to these coded panels (108) without publication of a definitive report. Thus, prospects for early availability of an immunoassay with specificity for an NAB antigen remain limited. Moreover, as mentioned earlier, RF, undetectable with conventional RF assays, has been shown to yield false-positive results in immunosays for NAB hepatitis antigens (110). Until additional tests of specificity have been described, reproducibility of any of these assays in several laboratories has been achieved, and one or more of these assays has been shown to identify donors implicated in the transmission of NAB hepatitis. Reports of the validity of these assays must be interpreted with caution.

**Delta Agent**

An intriguing discovery is that the recently described delta (δ) antigen associated with HBV infection (167) represents the immunologic marker of a defective transmissible agent other than HBV, which requires HBV helper function for its replication and expression (168,169). Thus, δ may represent an NAB hepatitis agent. In support of this possibility, the similarity of light microscopic features in δ-associated and NAB hepatitis (170). A recent report that the cytoplasmic tubular structures observed ultrastructurally in NAB hepatitis (51) are also observed in HBsAg carrier chimpanzees with acute δ infection (171) lends additional support to this hypothesis. Delta is detected, however, only in serum or liver of patients with HBV infection, not of patients with non-B hepatitis. Moreover, antisera that stain purported NAB antigen in hepatocyte nuclei are devoid of anti-δ (122). Therefore, whether or not δ antigen is the marker for an NAB agent associated with HBV, it is not related to the set of HBsAg-negative patients with NAB hepatitis defined originally in transfusion settings, and more recently in other epidemiologic situations, i.e., the variety of patients discussed in this review. Although some have postulated that one or more NAB hepatitis agents may resemble δ-like defective viruses that require the help of an HBV-like virus, data sufficient to support this possibility are lacking.
Putative Markers in Perspective

Why the agents of NANB hepatitis remain so difficult to identify conclusively with in vitro tests remains an enigma. It is likely that the concentration of circulating viral antigens and particles is very low, on the one hand, and the serum titer of NANB antibodies is relatively low on the other, factors that render immunodiagnosis unusually challenging. The possibility that a large proportion of, or even most, infected persons remain virus carriers after acute NANB hepatitis infection and fail to acquire antibody may explain why obtaining convalescent antibody-containing serum samples adequate for immunologic testing is so difficult. In fact, such an inadequate humoral immune response may be responsible for the high frequency of chronic hepatitis after acute NANB hepatitis and the probable high frequency of asymptomatic chronic carriers (172,173). Other factors that may contribute to the difficulty of identifying serologic markers and virus particles include the possibility that there are multiple NANB agents or that there are multiple antigen–antibody systems associated with a single or a limited number of agents. The high frequency with which nonspecific antibodies, unusual autoantibodies, and allobodies appear in patients with NANB hepatitis (100,107,109,110,159,160,174) also adds to the confusion. That viral antigens circulate masked within immune complexes is another possibility that would account for the difficulty of detecting an antigen–antibody system by standard immunologic techniques. As reported (105) and confirmed (175–180), circulating immune complexes can be detected in a large proportion of patients with NANB hepatitis, especially in those with chronic hepatitis, and in chimpanzees with chronic NANB hepatitis (23). Suh et al. (102) have shown, in addition, that immunoprecipitin lines associated with NANB hepatitis have properties of immune complexes. Characterization of these immune complexes has revealed the presence within them of an 80,000 dalton nonimmunoglobulin protein (177; Dietzsch JL, unpublished observations). Attempts to identify a viral antigen within these complexes, however, have been fruitless (178,179), and Penner et al. (175) have identified single-stranded DNA within circulating immune complexes from patients with NANB hepatitis. In all likelihood, the high frequency of circulating immune complexes in NANB hepatitis results from an impairment in normal Kupffer cell clearance function, reflected morphologically in pronounced activation of sinusoidal macrophages (34–36). Thus, these immune complexes probably contain nonvirus antigens (e.g., enteric and food antigens). Ultimately, new approaches, such as attempts to detect and clone nucleic acid, in conjunction with biochemical and biophysical characterization of the agent(s) in experimental animals, may be necessary to identify NANB hepatitis virus(es) definitively.

In summary, then, a variety of antigen–antibody systems and viruslike particles have been described in patients and experimental animals with NANB hepatitis. Some reports have been promising, and one or more of these putative NANB hepatitis systems may prove ultimately to be accepted universally. Currently, however, no serologic test is sufficiently reliable (182,183) to be an intermediate or specific to warrant its adoption as a screening or diagnostic test for NANB hepatitis. The only reliable indicator of NANB hepatitis remains infectivity in experimental animals.

Is Non-A, Non-B Hepatitis Related to Hepatitis B?

A number of observations described earlier suggest that NANB hepatitis may be similar to HBV. Non-A, non-B hepatitis and hepatitis B share several (but by no means all) clinical and epidemiologic features... and... in a proportion of chimpanzees with experimental NANB hepatitis, morphologic changes in the liver are indistinguishable from those of experimental hepatitis type B (34,35). Trepo and colleagues (15,16,63–61) have described HBV-like virions associated with DNA polymerase and antigen–antibody systems in NANB hepatitis that correspond to the surface, core, and e antigens of HBV. Based on the apparent similarities between the NANB antigens and particles with those of HBV and the apparent immunologic cross-reactivity between the core and e antigens of NANB hepatitis and HBV, Trepo and colleagues have postulated that NANB hepatitis is related to HBV and belongs in the HEPADNA virus group with HBV and hepatitis viruses of woodchucks, ground squirrels, and ducks. The fact that the presence of anti-HBc in donor blood is associated with a threefold increase in the risk of NANB hepatitis to blood recipients (181) and that anti-Hbc (as well as anti-HBs) can be found in donor blood given to recipients in whom NANB hepatitis developed (182,183), has been interpreted to suggest that HBV-like viruses are responsible for a substantial proportion of cases of posttransfusion hepatitis classified as NANB hepatitis (183). Cases of seronegative HBV infection have been described in which intrahepatic HBV antigens are demonstrable (184). HBsAg has been detected with monoclonal anti-HBs probes in a limited number of patients with hepatitis, including posttransfusion hepatitis, whose sera were negative for HBsAg when tested with conventional assays (185–187), and HBV DNA has been
detected in the livers of a limited number of patients with HBsAg-negative acute and chronic hepatitis and hepatocellular carcinoma (186,189).

Moreover, three groups of investigators claim to have identified HBV DNA in the livers of patients with chronic NANB hepatitis. Charnay et al. (190) detected integrated HBV DNA in 1 patient and free HBV DNA in a second of 5 patients with chronic NANB hepatitis in whom immunofluorescence tests for the intrahepatic “NANB antigen” of Vitiwiski et al. (62) were positive, but in whom all serum markers and liver antigens of HBV were absent. Figus et al. (191) examined the livers of six multiply-transfused thalassemic patients with HBsAg-negative chronic hepatitis and found integrated HBV DNA in all 6, as well as free DNA in 4 of the 6. Shafritz et al. (187) and Wands et al. (17) reported the detection of HBV DNA in NANB hepatitis serum samples in which apparent HBsAg reactivity was detectable with a monoclonal anti-HBs probe, but not with conventional assays relying on a polyclonal anti-HBs probe. In the report by Shafritz et al. (187), HBV DNA and a positive monoclonal RIA test for HBsAg were detected in the serum from 1 patient with posttransfusion hepatitis and in four blood donor serum samples, only one of which was implicated in transmitting hepatitis to a blood recipient. None of the five samples contained anti-HBc, anti-HBs, or HBsAg when tested with a conventional polyclonal assay. Wands et al. (17) described studies in which NANB hepatitis inocula were administered to 4 chimpanzees, 2 of which had anti-HBs (but not anti-HBc) before inoculation from an earlier HBV infection. All 4 had “antigenemia” detectable with the monoclonal anti-HBs RIA (but not a polyclonal RIA), and the 2 with prior anti-HBs, but not the 2 who were anti-HBs-negative, had HBV DNA detectable during the time of antigenemia. Only 2 of the chimpanzees had ALT elevations, which, curiously, preceded “antigenemia” by 24 and 42 days, respectively.) These studies were interpreted as evidence for HBV-related agents in NANB hepatitis. As Vyas and colleagues (192–194) have postulated, NANB hepatitis, in some cases, may be caused by viral agents that are genetically similar but antigenically distinct from HBV. Thus, theoretically, through mutations and rearrangements in DNA, HBV-like agents that share partial DNA homology with HBV, but which lack antigenic markers of HBV, may arise.

Although these findings are intriguing and provocative, evidence against the association between HBV and NANB hepatitis is considerably more compelling. On a simple clinical level, the two diseases are quite different (see Tables 1–5 in part I of this review) (195). In addition, although infection with HBV confers homologous immunity against reinfection with HBV, NANB hepatitis has been shown to occur in persons previously infected with and who had recovered from hepatitis B (1,48,190,197). In such cases, anamnestic responses to HBV markers have not been detected (1). Moreover, chimpanzees infected first with HBV are immune to reinfection with HBV, but are readily susceptible to NANB hepatitis infection, and chimpanzees infected first with NANB hepatitis are susceptible to a later infection with HBV (21). Even if an individual case designated as NANB hepatitis were a case of seronegative HBV infection, serial transmission of that infection should lead to HBV antigen or antibody expression in subsequent passage of the inoculum in experimental animals. On the contrary, studies of NANB hepatitis in experimental animals have shown beyond doubt that all serum and liver markers of HBV infection, including HBsAg, HBcAg, and marmosets, serial sera evaluated throughout the course of illness, convalescence, and complete recovery remain devoid of markers of new HBV infection. Recall, also, that marmosets are not susceptible to HBV infection, yet they can be infected with NANB hepatitis agents (24,25). Relatedness of HBV and NANB hepatitis is also rendered unlikely by the failure of hepatitis B vaccination to prevent NANB hepatitis (198), and by the finding that the NANB hepatitis agent transmitted in chimpanzees (3) and prevalent in patients with transfusion-associated hepatitis (52) is smaller than poliovirus (41,42), which is considerably smaller than HBV.

Although serologic markers and viruslike particles comparable to those of HBV have been described in NANB hepatitis (68), the following factors all argue against the suggested association between these two agents: (a) the absence of specificity of these tests when subjected to coded serum panels (44,108); (b) the absence of these markers, particles, or particle-associated DNA polymerase from sera and livers of experimentally infected chimpanzees (82,83) and humans studied by other investigators; (c) the failure to confirm the validity of these findings by at least one of the original investigators involved in their description (82,83); and (d) the marked difference in ultrastructural findings in the liver between hepatitis B and NANB hepatitis (51). The fact that donor blood with anti-HBc is more likely to transmit NANB hepatitis to recipients is no different than the association reported between anti-HBs in donor blood and the increased risk of NANB hepatitis to recipients (199)—both are probably a reflection of common features of segments of the donor population that predispose them to exposure both to HBV and NANB.
agents (172), just as there is an increased frequency of antibody to HAV (anti-HAV) in blood donors with serologic evidence of exposure to HBV (200). Moreover, all but 1 of the 11 anti-HBc-positive blood donors implicated in transmission of NANNB hepatitis in the reports of Cossart et al. (182) and of Vyay and Perkins (183) had anti-HBs (1 had HBsAg) in serum as well, indicative of HBV infection in the past (or present in 1 case), and none of the recipients of anti-HBc-positive blood included in those studies or in the larger Transfusion-Transmitted Viruses (TTV) Study (173,201) in whom NANNB hepatitis developed over expressed anti-HBc or any other HBV serum marker during or after NANNB hepatitis. In addition, others have failed to identify an increase in the risk of NANNB hepatitis in recipients of anti-HBc-positive blood (202).

Admittedly, there are occasional patients in whom expected HBV markers are absent in serum but present in the liver, especially in such groups as cirrhotics, hemophiliacs, homodilysi patients, immunosuppressed oncology patients, and those with hepatocellular carcinoma (186,188,203–207). Even among patients with HBsAg-negative liver disease who have HBV DNA in the liver or among those whose HBsAg activity is detected with monoclonal but not polyclonal anti-HBs probes, most have other serum markers of HBV or intrahepatic HBV antigens (17,185,186,188,189). For example, of 11 HBsAg-negative patients with chronic hepatitis or cirrhosis described by Brechot et al. (188) in whom HBV DNA was present in the liver, 8 of the 11 had other HBV serum markers and all 11 had intrahepatic HBV antigens. In contrast, patients and chimpanzees with carefully pedigreed and documented NANNB hepatitis have been shown to be free of serum and liver markers of HBV infection.

As for the monoclonal anti-HBs probes used to identify HBsAg reactivity in presumptively HBsAg-negative patients, including several with posttransfusion hepatitis, this approach has not been successful in identifying the same patients purported to have HBV-like markers by Trope and his associates (Trope C, personal communication), nor have these probes been used successfully to identify NANNB antigens in the livers of experimentally infected animals (Feinstein SM, personal communication). Furthermore, neither monoclonal anti-HBs probes (185–187) nor the tests for HBV-like antigens described by Trope et al. (88) have performed well on coded panels of serum from pedigreed cases of NANNB hepatitis and controls (44,108). The fact that the HBV-related serum marker detectable with a monoclonal anti-HBs probe appeared in the circulation of chimpanzees with experimental NANNB hepatitis (17) is no more compelling than the demonstration in experimentally infected chimpanzees of CEP (117), immunofluorescence (16,62,122), and EIA (18) antigens by other investigators. Each of these assays has performed poorly and proven nonspecific when subjected to validation on coded panels of serum (44,108). Moreover, the monoclonal anti-HBs RIA detects an “NANNB” antigen only when the same monoclonal IgM anti-HBs is used as both ends of the RIA sandwich, i.e., as the antibody bound to the solid-phase bead and as the tracer antibody probe. When monoclonal IgG anti-HBs probes are substituted for the radiolabeled monoclonal IgM anti-HBs probe in the sandwich RIA, “NANNB” reactivity cannot be detected (17). This is consistent with a recent analysis that suggests that the apparent HBsAg reactivity identified with a monoclonal IgM anti-HBs probe in HBsAg-negative serum is a low isoelectric point IgM with rheumatoid factor properties, i.e., a false-positive reaction (Zuravski VR Jr, personal communication).

The reports described above of HBV DNA in livers of patients and chimpanzees with acute and chronic NANNB hepatitis (17,187,190,191) do not necessarily indicate that HBV caused the episode of NANNB hepatitis. Integrated HBV DNA may have resulted from HBV infection in the past or may have been an artifact caused by contamination of the HBV DNA probe with nonviral DNA. Moreover, these reports of HBV DNA in livers of patients with NANNB hepatitis stand in contrast to other reports that failed to confirm these findings. Prince et al. (208) using the same technique and working in the same laboratory as Charnay et al. (190), studied liver tissue from 12 chimpanzees with acute and 2 with chronic experimentally induced NANNB hepatitis. In none of the 14 could DNA homologous with HBV DNA be detected. Even Trope et al. (15), who collaborated with Charnay et al. (190) in the study in which HBV DNA was detected in NANNB hepatitis liver tissue, has recently reported that he could not detect HBV DNA in liver from a chimpanzee with experimental NANNB hepatitis. Similarly, Monjardino et al. (209) were unable to detect sequences of HBV DNA in serum or livers of humans, chimpanzees, or marmosets with NANNB hepatitis. In yet another report, Fields et al. (210) showed that there was no homology with HBV DNA in serum from a chimpanzee with factor VIII-associated NANNB hepatitis (and no HBV-specific DNA polymerase activity in any of 12 acute-phase or any of six chronic-phase sera from chimpanzees with this type of NANNB hepatitis). Although these studies do not absolutely rule out areas of minor homology (209), they add to the weight of the evidence marshalled above against the relatedness of HBV and NANNB hepatitis.

Until adequate tests for NANNB hepatitis agents are...
available and NANB hepatitis virus(es) have been characterized definitively, or until tests for HBV-associated markers can be shown in prospective studies to identify donor units that would transmit NANB hepatitis, conclusions about the presence or absence of a relationship between NANB hepatitis agents and HBV cannot be made with absolute confidence (79,83,209). On the other hand, although some cases being labeled currently as NANB hepatitis may be misclassified, and really represent "serologically silent" HBV infections, and although additional studies will be necessary to determine whether minor areas of genomic homology or whether small numbers of shared epitopes occur between HBV and some or all NANB hepatitis virus(es), most cases of NANB hepatitis, current evidence suggests, bear no major structural or immunologic relationship to HBV.

Prevention

Studies designed to test the efficacy of preventive measures are handicapped by the inability to document serologically sources of infection, susceptibility of contacts, or the presence of antibodies to NANB hepatitis antigens in globulin preparations. Nevertheless, several indirect methods to limit the spread of NANB hepatitis, primarily among blood recipients, have been suggested.

Proposed Interim Screening Tests

In the absence of a specific serologic screening test for NANB hepatitis, are there any features of donor blood that can be used to predict the likelihood of its transmitting NANB hepatitis to recipients? As detailed earlier (195), the most effective measure introduced to reduce the frequency of post-transfusion hepatitis is elimination of blood from commercial donors, which is much more likely to transmit NANB (or type B) hepatitis than blood obtained from volunteer donors. A history of jaundice in the past, prior transfusion, and donor age, however, do not correlate with the likelihood of transmitting NANB hepatitis (211).

Among the non-virus-specific serologic and biochemical markers in donor blood that have been proposed to correlate with infectivity for NANB hepatitis are anti-HBs (189), anti-HBc (66,161,163), carcinoembryonic antigen (CEA) (212,213), bile acids (213,214), and alanine aminotransferase (ALT or SGPT) (108,173,201,213–216). In one study by Conrad et al. (199), the frequency of NANB hepatitis after transfusion was greater among recipients of blood containing anti-HBs than among recipients of anti-HBs-negative blood. On the other hand, Sectl et al. (172) have shown that the apparent higher incidence of hepatitis in recipients of anti-HBs-positive blood was not related to the presence of anti-HBs per se, but to the higher frequency of anti-HBs in commercial blood. Patients who had received blood containing anti-HBs and who had a higher frequency of NANB hepatitis were more likely to have received several units of commercial blood than patients who had received anti-HBs-negative blood and who had a lower frequency of hepatitis. As stated above, anti-HBs in such donor blood with an increased risk of transmitting NANB hepatitis appears to be a reflection of the enhanced exposure to both HBV and NANB hepatitis in a subsegment of the population, and the same argument can be advanced to counter the claim that anti-HBc in donor blood is a marker predictive of the development of NANB hepatitis in recipients (183). In fact, the correlation observed by Conrad et al. (199) between donor anti-HBs and the NANB hepatitis risk in the recipient may have been related to reliance in that study on military blood donors, who are comparable to civilian commercial donors in terms of hepatitis risk (217,218). In any event, others have failed to confirm the association between anti-HBs (201,217,219,220) in donor blood and enhanced risk of hepatitis in recipients.

As noted earlier, preliminary data presented by the TTV Study Group indicated that recipients of at least one unit with anti-HBc were three times more likely to acquire NANB hepatitis after transfusion than recipients of blood that was negative for anti-HBc (181). Theoretically, therefore, anti-HBc could serve as an indirect screening test for donor units that are likely to transmit NANB hepatitis. In the TTV Study, however, the corrected efficacy of anti-HBc as a screening test was slightly less than that of ALT (see following discussion), and the number of blood units lost was twice what would be lost if ALT were used (Stevens CE, personal communication). Thus, anti-HBc screening would double the number of units lost without any advantage over ALT screening in preventing recipient hepatitis.

Gitnick and colleagues (212,213) reported a higher frequency of NANB hepatitis in recipients of blood with elevated levels of CEA and serum bile acids. These markers appear to be too nonspecific. Although bile acids and CEA may become elevated in patients with NANB hepatitis (101,212,213,221), subsequent studies have failed to confirm the usefulness of these markers in predicting the NANB hepatitis infectivity of donor blood (214). The nonspecific marker that has received the most attention is ALT (108,173,201,213–216). In recipients studied by the TTV Study Group (173), the NANB hepatitis attack rate was 37.5% among recipients of multiple units of which at least one had an elevated ALT level (≥45
IU), compared with an annual attack rate of only 7.1% for recipients of blood with ALT levels <45 IU. In recipients of a single unit of blood, a donor ALT of ≥60 IU was associated with a 50% NANB hepatitis attack rate in the recipient. Furthermore, in few patients receiving 2 units of blood with an ALT level ≥45 IU, the NANB hepatitis attack rate was 91%. The relationship between donor ALT and hepatitis in recipients was independent of both transfusion volume and blood source (commercial vs. volunteer) (173). Comparable figures were reported by Alter et al. (108,215), who found an NANB hepatitis frequency of 29% in recipients of at least 1 unit with an ALT >53 IU (20.7 cases/1000 units transfused), compared with a frequency of only 9% in recipients of units with ALT levels ≤53 IU (7.6 cases/1000 units).

Despite these alarming figures, many questions have been raised about the value of ALT screening to prevent NANB hepatitis after transfusion (222–233). First, the characteristics of ALT as a screening test are not impressive. Based on data from the TTV Study (173) in single unit transfusions and an ALT cutoff of 45 IU, the sensitivity of the test is only 26%. That is, ALT screening would identify only 26% of donor units that can transmit NANB hepatitis; the false-negative rate or frequency of infectious units missed would be 74%. Although the specificity of the test (absence of infectivity when the ALT level is <45 IU) is high, 97% (only a 3% false-positive rate among all blood donors), the predictive value of the test (likelihood the blood unit will be infectious when the ALT level is elevated) is only 42%, or, conversely, the false-alarm rate is 58%. In other words, almost two out of every three units with an elevated ALT level will not transmit NANB hepatitis. Because elevated ALT values are found in as many as 5%–6% of all blood donors in some populations (223,225), withholding all these donor units would eliminate a sizeable enough proportion of blood donors to compromise the national blood supply. What is worse, many of these units would not have caused hepatitis in recipients; ALT levels have been shown to be elevated more frequently in such groups as men, those who drink alcohol, persons being treated with antihypertensive or diuretic medications, persons in the fourth decade of life, those who are married, those of lower socioeconomic status, and nonwhites (224–227). Moreover, in one study, only 40% of donors with elevated ALT levels had persistently elevated levels when retested at 6 mo (225). Thus, we run the risk of deferring many donors whose blood is unlikely to be infectious and whom we have to notify of this abnormal biochemical test. No guidelines exist for advising these donors or for determining how long to defer them. Furthermore, handling of serum affects ALT activity (226), and standardization of ALT testing among laboratories is poor.

Still, as estimated by Alter et al. (215), eliminating blood donor units with ALT levels >2.25 SD above the mean log for normals would be effective in preventing 29% of all NANB hepatitis cases after transfusion at a loss of donor units of only 1.6%. Nationally, this translates into prevention of ~100,000 cases each year. Moreover, cost-effectiveness analysis shows that introduction of ALT screening, if it could be accomplished at a cost of ~$2/unit, would be cost-saving: approximately $400 would have to be spent on ALT testing to prevent a single case of acute NANB hepatitis, a small price to pay considering the much higher direct medical costs and the indirect costs (e.g., time lost from work) of a case of hepatitis (222). If prospects for a specific test for NANB hepatitis were bright, this interim test might not be worth considering. On the other hand, an intensive search for serologic markers begun a decade ago has yet to bear fruit, and as many as 5–10 yr may pass before a specific, sensitive, screening test is developed and introduced into practice. Therefore, despite the poor sensitivity and predictive value of the test, and despite the difficulties and questions generated by a policy of screening, ALT screening may be warranted until NANB-specific tests become available. Currently, no policy to initiate ALT screening has been adopted officially (233), but several large centers are screening and withholding blood with high ALT levels, and prospective studies are planned to evaluate the effect on the frequency of posttransfusion hepatitis of ALT screening and withholding blood units with elevated levels.

Passive Immunoprophylaxis

Conflicting reports have appeared about the efficacy of immune globulin (IG) in preventing transfusion-associated hepatitis (234), but in only three studies of relatively recent vintage have NANB and type B hepatitis been distinguished (172,235,236). Even among these three studies, conflicting findings have emerged. Kahn et al. (235) reported that, compared to placebo, IG was not effective in reducing the incidence of icteric, anicteric, or prolonged hepatitis among patients who had received transfusions associated with cardiovascular surgery. In this study, 76% of the blood was derived from volunteer donors and the remainder from commercial sources; all blood had been screened for HBsAg. Two 10-ml doses of IG were given, with the first on day 7 ± 3 after surgery, and the second on day 30 ± 5. In contrast, Knodell et al. (236), who studied cardiac surgery patients who had received transfusions at
two Army hospitals, reported that, compared with placebo, a single 10-ml injection of globulin administered before surgery decreased the incidence after transfusion of icteric, anicteric, and chronic hepatitis (237). Although blood was screened for HBsAg and considered of volunteer origin, the blood was derived from military volunteers, who have been shown to be comparable to civilian commercial blood donors in terms of hepatitis risk (217,218). The third study, which, like the others, was randomized, double-blind, and placebo-controlled, was conducted by Seeff et al. (172) on Veterans Administration hospital patients who received small volume transfusions. Unlike cardiac surgery patients in the other two studies, who received a mean of 12 (236) to 18 (235) units of blood, patients in the Veterans Administration study received a mean of only 3.3 units. Ten milliliters of IG or placebo were administered within 96 h of the first transfusion, and an additional 10 ml 28 ± 3 days later. In this study, significantly fewer cases of icteric NANB hepatitis occurred in the IG-treated group, but the incidence of total (icteric plus anicteric) hepatitis and chronicity was the same in both groups. The beneficial effect of IG on icteric hepatitis, however, was confined to patients receiving ≥3 units of commercial blood; there was no discernible difference in icteric hepatitis between IG-treated and placebo-treated participants who had received volunteer blood. In retrospect, then, the effectiveness of IG reported by Knodell et al. (236,237) in reducing the frequency of both acute and chronic hepatitis after transfusion may have resulted from the use of the military equivalent of commercial blood donors. Moreover, the 17% incidence of transfusion-associated hepatitis during the study was well above the incidence expected for volunteer blood but well within the range expected for commercial blood. In addition, as Goldfeld et al. (217) have shown, chronicity of posttransfusion hepatitis occurs more frequently after commercial than after volunteer blood transfusions. Furthermore, biochemical follow-up in the Army study (236) was less intensive than in the other two studies and may have missed a sizeable proportion of anicteric cases, resulting in an artificial demonstration of benefit among more severe cases.

These three studies are not exactly comparable, and, theoretically, the remarkable benefit identified in the study of Knodell et al. (236,237) could have resulted from the administration of IG before rather than after transfusion or from use of an IG lot with higher levels of antibodies to NANB hepatitis agent(s). The possibility that IG may be of some benefit in preventing or attenuating NANB hepatitis requires additional consideration, especially in light of a report that IG for intravenous administration (venoglobulin), when added at a dose of 250 mg to each unit of blood 1 h before transfusion, reduced the frequency of NANB hepatitis by >60% (5% in venoglobulin-treated vs. 13.7% in placebo-treated patients) (cited in 234). When serologic tests for NANB hepatitis infection become available, the potency of IG and its efficacy in preventing transfusion-associated hepatitis can be reevaluated more intelligently.

Thus, despite the apparent effectiveness of IG in decreasing the severity of NANB hepatitis after transfusion of commercial blood, and because the same effect can be achieved by converting from commercial to volunteer blood, IG is not recommended for routine prophylaxis of posttransfusion NANB hepatitis (234). The only effective way currently available to prevent transfusion-associated NANB hepatitis is to eliminate the use of commercial blood donors (and, perhaps, to screen donor blood for ALT).

Other Measures to Prevent or Minimize Non-A, Non-B Posttransfusion Hepatitis

Avoidance of transfusions unless absolutely essential, keeping transfused blood and blood products to a minimum, avoiding pooled blood products and concentrates, reliance on autologous blood, and programs to identify and defer donors implicated definitively in the transmission of NANB hepatitis all contribute to minimizing the risk of hepatitis after transfusion (228). If preliminary reports suggesting the value of heating (26,39) or of β-propiolactone combined with ultraviolet irradiation in inactivating NANB hepatitis agent(s) (40) can be confirmed, such treatment of blood products may reduce the risk of hepatitis to recipients (although β-propiolactone is considered carcinogetic). Debate continues over the value of using frozen-deglycerolized red blood cells to eliminate or reduce the risk of posttransfusion hepatitis. Whereas early studies suggested that the risk of hepatitis after transfusion of frozen-deglycerolized blood was reduced (236), a subsequent study failed to show any advantage of this type of blood (239). In addition, conversion from commercial to voluntary blood donors has an impact that outweighs profoundly the impact of the use of frozen or washed red cell transfusions on the incidence of hepatitis (239). Because freezing and deglycerolizing involves substantial washing of red cells, the freeze-thaw process may dilute out contaminating viruses and may reduce the risk of transmitting hepatitis viruses by transfusion but is unlikely to be completely effective in preventing virus transmission. Finally, one randomized, double-blind, placebo-controlled study has been completed in which the efficacy-of
vitamin C therapy for the prevention of transfusion-associated hepatitis was assessed; however, no significant difference in the frequency of hepatitis after transfusion or the clinical course of hepatitis was observed between the treated and untreated groups (240).

Non-Transfusion-Associated Hepatitis

There are no specific guidelines for prevention of NANB hepatitis after needlestick, sexual contact, family contact, institutional contact, or neonatal exposure. Although data about the efficacy of IG in preventing transfusion-associated NANB hepatitis are equivocal, conceivably IG might be more effective in the setting of a smaller inoculum than an entire unit of transfused blood. Limited observations support this hypothesis. Gayer et al. (32) noted that in household contacts of patients involved in a plasmapheresis unit-associated NANB hepatitis outbreak, secondary cases occurred in 2 of 34 patients (5.9%) who did not receive globulin, but in none of 5 who did. Similarly, Simon et al. (81) evaluated the role of hepatitis B immune globulin (HBIG), probably administered in an attempt to control hepatitis B, in preventing NANB hepatitis in a hemodialysis unit. Among 32 patients observed for the first 9 mo after the initiation of hemodialysis, NANB hepatitis occurred in only 1 of 22 globulin-treated patients (4.5%) compared with 5 of 10 non-globulin-treated patients (50%). Unfortunately, these analyses were based on retrospective observations, comparability of risk factors between treated and nontreated groups was not described, and the numbers were too small to support firm conclusions.

Given the resemblance between the epidemiology of type B hepatitis and the most common types of NANB hepatitis, some authorities recommend prophylaxis with 0.06 ml/kg of IG, intramuscularly, for percutaneous, sexual, and perinatal exposure to well-documented or suspected cases of NANB hepatitis (234). Because less intense types of exposures are unlikely to transmit NANB hepatitis, family members other than sexual partners, as well as institutional contacts, are probably not at substantial risk and need not receive prophylaxis. On the other hand, IG is inexpensive and extraordinarily safe, and, early in the course of acute hepatitis, a rapid distinction between type A and NANB hepatitis cannot be made readily. Under these circumstances, it is prudent to administer IG to household and institutional contacts of patients with acute NANB hepatitis as though one were dealing with HAV exposure. For the water-borne type of NANB hepatitis, the effectiveness of IG prophylaxis remains to be determined.

Summary and Conclusions

Suggestions from earlier studies of hepatitis, as well as more recent serologic studies and transmission studies in volunteers and chimpanzees, provide compelling evidence for the existence of human hepatitis viruses besides HAV and HBV. The type of NANB hepatitis encountered most frequently is epidemiologically similar to type B hepatitis. Transmitted predominantly by transfusion and percutaneous inoculation, NANB hepatitis accounts for >90% of posttransfusion hepatitis but, like hepatitis B, can be transmitted by nonpercutaneous routes. Approximately 15%-30% of sporadic hepatitis cases are attributable by serologic exclusion to NANB hepatitis agents, and recognition has emerged recently of an epidemic form of water-borne NANB hepatitis, like hepatitis A, transmitted by the fecal-oral route. Clinical features of the predominantly percutaneously transmitted forms of NANB hepatitis are similar to those of type B hepatitis but tend to have shorter incubation periods, to be less severe during acute illness, and to lead more frequently to chronic hepatitis. After transfusion, as many as 40%-80% of patients with acute NANB hepatitis will be left with chronic elevations of serum aminotransferase activity, often in a fluctuating pattern; histologic features of the liver in a majority of patients with transfusion-associated chronic NANB hepatitis are consistent with chronic active hepatitis, and ~10%-20% of chronic cases eventuate in cirrhosis. In most such cases of chronic active hepatitis, however, the disease tends to resolve slowly without therapy, but a small proportion of patients experience a rapidly progressive illness. The frequency of chronic liver disease after sporadic cases, in which the inoculum is unlikely to be as large as that in a transfused blood product, is lower (on the order of 10% or less), and chronic liver disease has not been recorded after the water-borne, epidemic type of NANB hepatitis. Both epidemiologic observations and studies of infectivity suggest that there is an asymptomatic chronic NANB hepatitis carrier state, and its frequency in the population appears to be many-fold higher than the frequency of the hepatitis B carrier state. Despite successful transmission of NANB agents to chimpanzees and marmosets, and a plethora of reports of promising putative NANB antigen–antibody systems, ultrastructural changes, and viruslike particles, no such system or virus has been identified that fulfills accepted serologic criteria for a specific causal association with NANB hepatitis. One or more of the serologic markers described more recently may prove to be virus-specific, but additional validation will be necessary. On the other hand, cross-challenge studies in chimpanzees have established con-
clusively that there are at least two different percutaneously transmitted NANB agents. Although administration of Ig may reduce the severity of hepatitis after transfusion of blood from commercial sources, currently, the most effective way to diminish the frequency of transfusion-associated NANB hepatitis is to rely exclusively on blood from volunteer donors. Until specific serologic tests are identified, screening donor blood for serum ALT and withholding units with elevated levels may allow elimination of approximately one-third of NANB posttransfusion hepatitis cases; however, pending additional study, no such screening policy has been adopted. The rapid, recent progress in our understanding of NANB hepatitis notwithstanding, the highest priority in the study of this disease is additional investigation to identify virus-specific serologic markers, which will be instrumental in demonstrating and characterizing the virus agent(s) and adding precision to our understanding of their biology, epidemiology, and clinical features.

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