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THE AGENTS OF NON-A, NON-B VIRAL HEPATITIS

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Recent studies have provided physicochemical and electron microscopic evidence for the existence of two distinct agents of posttransfusion non-A, non-B (NANB) hepatitis. One of these agents is chloroform-resistant and is not associated with the formation of unique ultrastructural structures in infected liver. The other agent is $CHCl_3$-sensitive, induces the formation of characteristic hepatocyte cytoplasmic tubules, and interferes with concurrent HAV or HBV infection in experimentally inoculated chimpanzees. The tubule-forming agent (TFA) has also been shown to pass through an 80 nm capillary pore membrane filter, suggesting that it is a small enveloped (or lipid-containing) virus. The TFA can also be recovered from low titer ($\leqslant 10^5$ infectious doses/ml) chronic-phase chimpanzee plasma by use of a multi-step purification procedure that assumes the agent is a small enveloped RNA virus with an approximate buoyant density of 1.24 g/cm^3 and a sedimentation coefficient of 200–280 S. The apparent lack of nucleic acid homology between the NANB-TFA and HBV further suggests that the NANB-TFA is either Togavirus-like or belongs to another or as yet undefined class of RNA or DNA virus.

non-A, non-B viral hepatitis chloroform-resistant agent $CHCl_3$-sensitive agent

INTRODUCTION

The term non-A, non-B (NANB) hepatitis has been used for the classification of liver disease that is not caused by the commonly recognized agents, namely, HAV, HBV, delta, CMV, EBV, or the more 'exotic' hepatotrophic viruses, Ebola, Lassa, Marburg, and Rift Valley Fever. In recent years, in fact, it has even become necessary to distinguish between those NANB hepatitis agents that are primarily transmitted by contaminated blood and blood products in contrast to those that are associated with fecally-contaminated drinking water and large-scale epidemics.

The following overview briefly describes the pathogenetic features of posttransfusion NANB hepatitis and discusses the nature of the putative etiologic agents.

PATHOGENESIS OF DISEASE

Approximately 5 to 10% of transfused individuals in the U.S. develop acute NANB hepatitis that is generally symptomatically mild and anicteric (75%) in nature. How-

ever, in sharp contrast to hepatitis B infection where up to 10% of patients may develop chronic liver disease, NANB-posttransfusion hepatitis (PTH) characteristically leads to persistent or intermittent liver enzyme (ALT) elevations. In one NIH study, Alter et al. (1982) reported that 68% of 75 prospectively followed PTH cases had persistent or intermittent ALT elevations for more than 1 yr and most for more than 3 yr. In spite of the apparently benign course of acute NANB-PTH, histologic follow-up of NANB patients with persistent ALT elevations has revealed that chronic active or persistent hepatitis frequently develops. Furthermore, of 72 NANB-PTH patients undergoing liver biopsy (composite of 6 PTH studies), cirrhosis was histologically confirmed in 22% (Alter, 1984). Thus, infection with at least one agent of NANB-PTH appears to lead to chronic sequelae in a significant proportion of cases.

EVIDENCE THAT NANB-PTH IS CAUSED BY A VIRUS-LIKE AGENT

The virus-like nature of the etiologic agent(s) of NANB-PTH is strongly supported by several lines of experimental and retrospective evidence, including the transmissibility of disease in humans and/or chimpanzees by intravenous inoculation of infected blood, blood fractions, or purified chronic-phase plasma preparations (Alter, 1978; Tabor, 1978; Bradley, 1979, 1980, 1981, 1983a; Wyke, 1979; Yoshizawa, 1980). Infectivity associated with at least one of the agents of PTH can be destroyed by (1) treatment with $CHCl_3$ (Bradley, 1983a; Feinstone, 1983), (2) treatment with 1 : 1,000 formalin (Tabor, 1980), and (3) heating at 100°C for 60 min (Yoshizawa, 1982) or 60°C for 10 h (Tabor and Gerety, 1982). More recent studies have also shown that the $CHCl_3$-sensitive agent will pass an 80 nm filter and that it can be recovered from chronic-phase plasma by use of a procedure suitable for purification of small enveloped viruses (Bradley, in press). Finally, the hepatocyte ultrastructural changes observed in chimpanzees infected with at least one major agent of NANB-PTH are identical or similar to those associated with the in vivo replication of certain classes of RNA (but not DNA) viruses (see below).

EVIDENCE FOR THE EXISTENCE OF TWO OR MORE DISTINCT AGENTS

The notion that NANB-PTH is caused by a collection of serologically unrelated hepatotrophic agents derives from (1) the observation of multiple attacks of hepatitis in patients transfused with blood products (Mosley, 1977; Hruby and Schauf, 1978; Norkrans, 1980); (2) the occurrence of both long and short-incubation period disease in transfused patients (Craske, 1975; Aach, 1978; Guyer, 1979); (3) the observation of unique or distinct ALT-patterns in infected patients (Tateda, 1979); (4) results of cross-challenge studies in chimpanzees experimentally infected with proven-infectious materials, including blood and blood products (Bradley, 1980; Hollinger, 1980; Tsiquaye, 1980; Yoshizawa, 1981); (5) the appearance of unique or distinct ultrastructural changes in infected chimpanzee hepatocytes (Shimizu, 1979; Yoshizawa, 1981;

Bradley, 1983a); and (6) the observation of multiple and sequential episodes of disease in chimpanzees following inoculation of physicochemically distinct NANB hepatitis agents (Bradley, 1983a).

It now appears that correct interpretation of some of the earlier chimpanzee cross-challenge study results may have been confounded by the effects of either persistent infection or viral interference. In the former case, some second bouts of disease in experimentally infected chimpanzees may have been due to exacerbation of chronic underlying infection rather than induction of nascent disease due to inoculation of a second presumed etiologic agent of PTH. On the other hand, apparent protection of a previously NANB-infected chimpanzee from infection with a second challenge inoculum (and distinct agent) could simply be due to viral interference rather than the presence of homologous, neutralizing antibody. In regard to the latter possibility, it is also important to note that homologous reinoculation of previously infected chimpanzees cannot necessarily be used as a method for the identification of animals with neutralizing (protective) antibodies, since reinoculation of persistently infected chimpanzees would, in all likelihood, not induce a second bout of disease. Our experience with NANB-infected chimpanzees during the past 7 yr has shown that all three conditions may be operative and that evidence for the existence of two or more distinct agents of NANB-PTH should derive from the use of physicochemically defined inocula (Bradley, 1983b). Since persistence of disease and/or viremia is probably the rule rather than the exception in infected individuals, much of the aforementioned evidence for the existence of multiple NANB agents should now be considered presumptive.

PERSISTENCE AND RECRUDESCENCE OF DISEASE

Chimpanzee transmission studies conducted in our laboratory have provided unequivocal evidence for the occurrence of long-term viremia and disease in experimentally inoculated animals. In fact, histologic examination of serial liver biopsy specimens from a total of 10 NANB infected chimpanzees revealed in all evidence of persistent liver disease as long as 5 yr after inoculation (Bradley, 1981, 1984). It is important to note that only 6 (or 60%) of these animals had persistent or intermittent elevations in ALT activity (with concurrent EM changes) suggestive of continuing liver dysfunction. This observation suggests that the presence of hepatic lesions may be a more sensitive marker for persistent NANB hepatitis than either elevated ALT activity or hepatocyte ultrastructural alterations. Persistent viremia was also demonstrated in six NANB-infected chimpanzees up to 6 yr after infection by inoculation of naive chimpanzees with chronic-phase plasmas obtained from these animals (Bradley, 1984a). It is interesting to note that plasma obtained from 4 animals with normal ALT activity, and lacking hepatocyte ultrastructural evidence of infection (i.e., tubules), were also infectious (Bradley, 1981, 1984). These findings are not surprising, particularly in view of the projections of Gerety et al. (pers. comm.) and Alter et al. (1982) that

approximately 70% of NANB-PTH would not be prevented by screening of blood donors for elevated ALT activity.

Related to the above finding of persistent disease in experimentally infected chimpanzees are our more recent observations that elevations in ALT activity can spontaneously occur, even after prolonged periods of normal liver enzymes (Bradley, 1982). For example, two chimpanzees acutely infected with a factor-VIII derived tubule-forming agent (TFA) developed typical biochemical and hepatocyte ultrastructural evidence of NANB-PTH. One animal appeared to have resolving hepatitis, as indicated by the normalization of serum ALT activity and loss of characteristic hepatocyte cytoplasmic tubules. However, 2 yr after inoculation, elevated ALT activity and hepatocyte tubules reappeared. Plasma obtained from this animal 4 yr after inoculation was shown to cause NANB hepatitis in a recipient chimpanzee. In contradistinction to the first animal, the second chimpanzee demonstrated low but significant and persistent elevations in ALT activity accompanied by the presence of hepatocyte tubules up to 20 mth after inoculation. This animal then had three episodes of severe disease during the following 40 mth in which elevations in ALT activity were found to be closely paralleled by the proliferation of smooth endoplasmic reticulum (SER) and hepatocyte tubules.

Our combined findings suggest that normalization of ALT activity and disappearance of hepatocyte tubules in NANB-infected chimpanzees do not necessarily signal recovery from disease. Furthermore, our results also strongly imply that some second bouts of NANB hepatitis in chimpanzees may actually be recrudescence of disease due to chronic underlying infection. If there are any parallels between the course of NANB hepatitis in chimpanzees and humans, it would appear that total resolution of disease may occur in a low proportion of infected individuals.

VIRAL INTERFERENCE

The phenomenon of viral interference has recently been documented in chimpanzees simultaneously infected with HBV and NANB or HAV and NANB (Bradley, 1983b; Brotman, 1983; Tsiquaye, 1983). Studies in our laboratory have revealed a profound effect of persistent NANB hepatitis infection on superinfection with HAV. Neither of two NANB-infected chimpanzees (both with persistent viremia and persistently or intermittently elevated ALT activity) developed additional elevations in ALT activity when challenged with proven-infectious HAV. In addition, both challenged animals demonstrated a delayed anti-HAV antibody response (28 and 43 days after inoculation) when compared to control chimpanzees (14 days after inoculation) who had received the same inoculum. Furthermore, neither superinfected chimpanzee was shown to have detectable HAV antigen by IF in 'acute-phase' liver biopsy specimens; all daily stool specimens, with one possible exception in one animal, were also negative by RIA for HAV Ag. More recently, we have also found that chimpanzees with biochemically and electron microscopically resolved NANB-PTH (> 1 yr)

may also demonstrate interference with HAV superinfection. This finding indicates that even presumed low-level replication of a NANB tubule-forming agent is sufficient to cause significant interference with at least one other hepatotrophic virus. Finally, we and others (Bradley, 1983b; Tsiquaye, 1983) have also shown that acute NANB-PTH also interferes with the replication of HBV in HBsAg carrier chimpanzees, as judged by the decrease in surface antigen titer and serum HBV DNA polymerase activity during the acute-phase of NANB disease. Coinfection of chimpanzees with HBV and the NANB-TFA has also been found to delay, moderate, or obviate the appearance of serologic markers of HBV infection (Brotman, 1983; G. Dolana, pers. comm.). It is now clear that acute and persistent NANB-PTH infections are capable of interfering with at least two different hepatotropic viruses, however, the mechanisms or effectors responsible for this phenomenon have not been identified.

ULTRASTRUCTURAL CHANGES

One of the most unusual, and perhaps revealing, aspects of experimental NANB-PTH in chimpanzees is the finding of unique ultrastructural alterations in hepatocytes of acutely and persistently infected animals (Shimizu, 1979; Bradley, 1980, 1981, 1984; Pfeifer, 1980). These changes are primarily confined to hepatocyte cytoplasm (Fig. 1) and include the formation of peculiar convoluted membranes (ERc), 150 to 300 nm diameter tubules comprised of double-unit membranes enclosing an as yet uncharacterized osmiophilic substance, and dense reticular inclusion bodies. Most of these structures appear to result from proliferated smooth endoplasmic reticulum (SER) in response to infection with the NANB-TFA (Fig. 2). Bundles of granular microtubules may also be observed in some infected hepatocytes; these structures are dissimilar to the more commonly described smooth microtubules in that they appear to be comprised of 25 nm diameter stacked disks (Fig. 3). The dense reticular inclusion bodies referred to above are fibrillar–granular masses of highly convoluted, densely stained materials that may, in fact, be comprised of microtubules coated with an amorphous, osmiophilic substance. Many of these structures contain multiple foci with radiating, strand-like attachments to the surrounding ER (Fig. 4). These latter structures are somewhat similar to the viroplasmic foci, or virus factories, described for mouse hepatitis virus, an RNA-containing coronavirus (David-Ferreira and Manaker, 1965) or influenza virus (Compans and Choppin, 1973). Although no convincing evidence for a virus-specific association of nucleic acid (RNA) with the NANB dense reticular inclusion bodies has been documented, it is worth noting that one group of investigators (Shimizu et al., pers. comm.) has found that EBV-transformed peripheral lymphocytes from a NANB-infected chimpanzee produce an IgM antibody that specifically binds to these structures. It is presently unclear whether this antibody is recognizing a virus- or host-specific antigen.

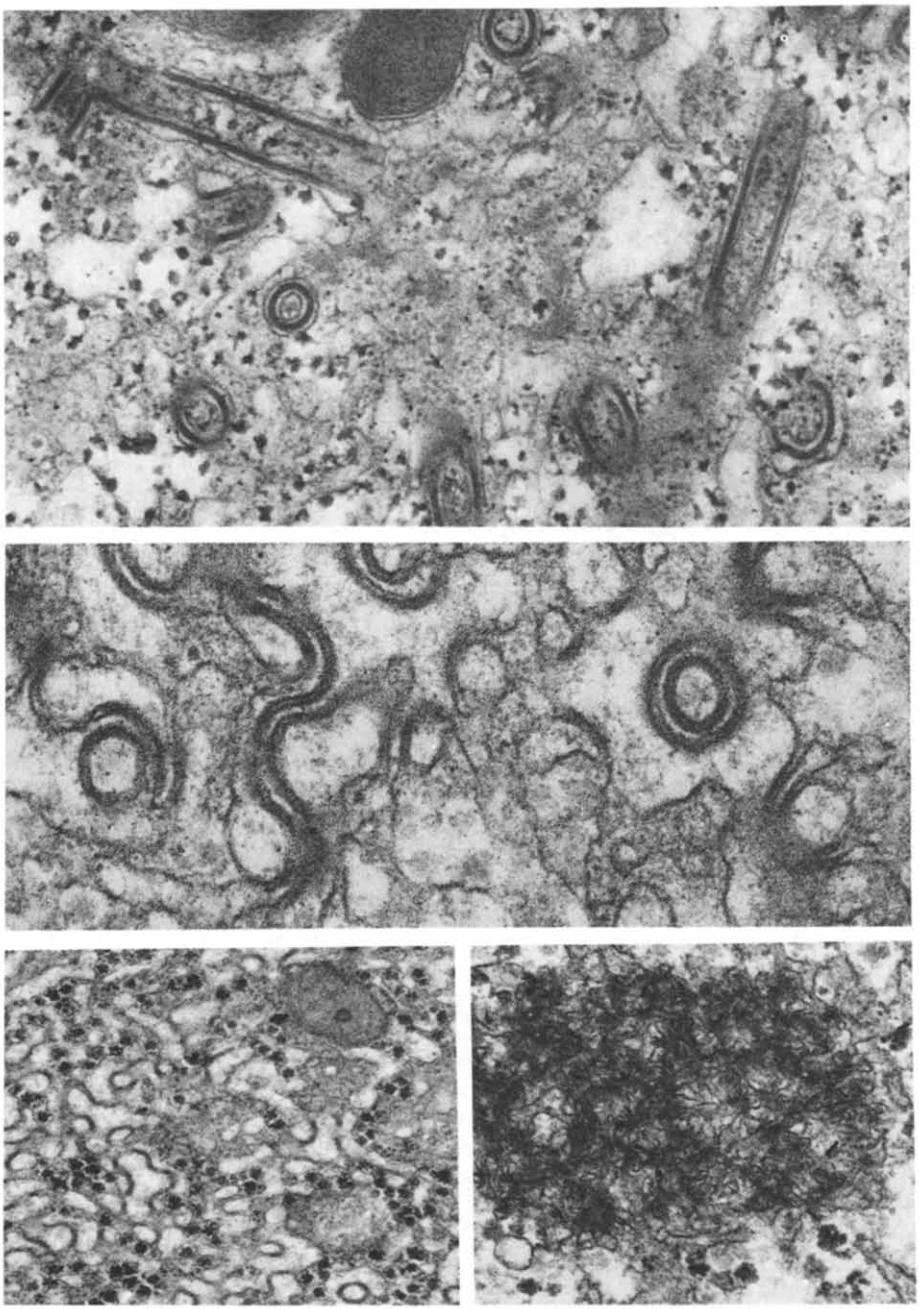

Fig. 1. Characteristic ultrastuctural changes in the cytoplasm of chimpanzee hepatocytes infected with a tubule-forming NANB agent. Upper: tubular structures derived from proliferated SER (× 38,500). Middle: paired membranes (ERc) consisting of cisternae of the ER in close apposition (× 79,830). Lower left: larger field showing extent of ERc (× 27,550). Lower right: dense reticular inclusion body with dense foci and radiating fibrillar strands (× 49,650).

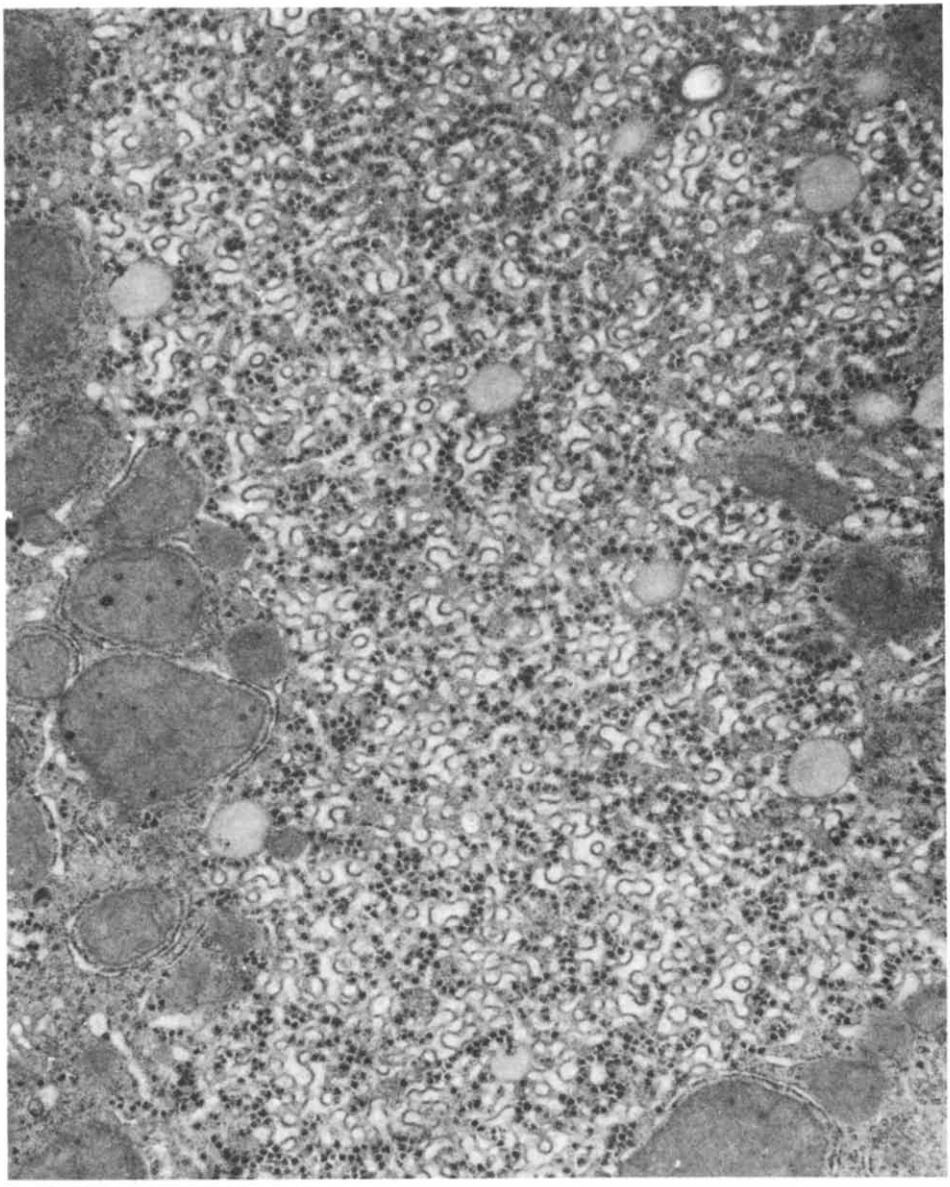

Fig. 2. Extensive proliferation of convoluted membranes (derived from SER) in an immunosuppressed chimpanzee (Bradley, 1984; × 18,850).

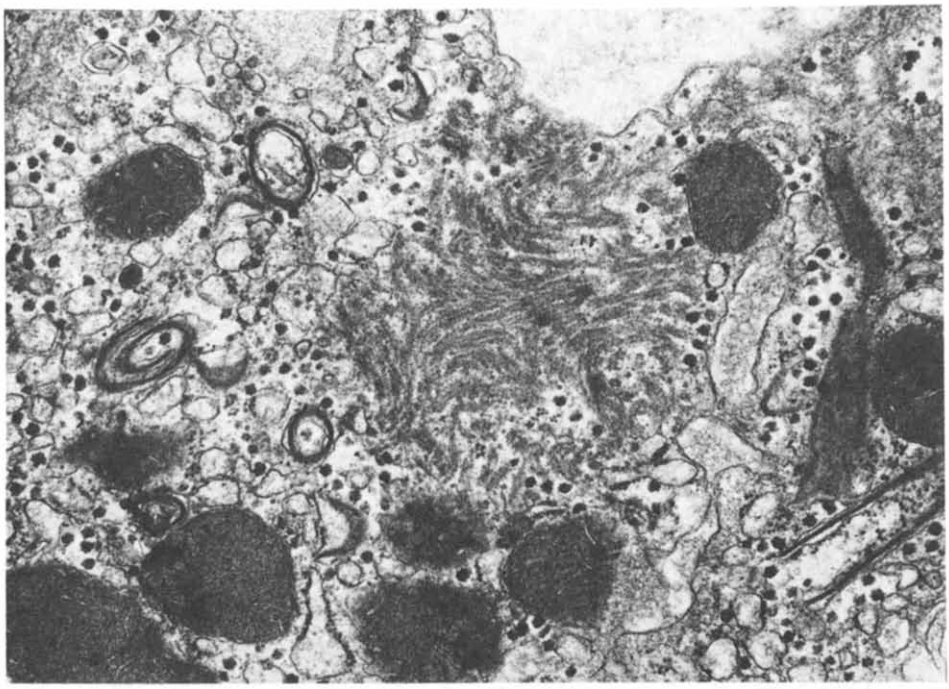

Fig. 3. Bundles of granular microtubules (center of photo) in chimpanzee chronic-phase NANB hepatocyte cytoplasm (× 25,350).

VIRUS-LIKE PARTICLES ASSOCIATED WITH NANB HEPATITIS

More than 14 different virus-like structures have been described for NANB hepatitis, yet none has been irrefutably linked to the transmission of disease. The putative virus-like particles (VLPs) were shown to range in diameter from 22 nm to 70 nm and include some that were morphologically similar to picornaviruses (Bradley, 1979; Yoshizawa, 1980), hepadnaviruses (Hantz, 1980), and Togaviruses (Coursaget, 1979). More recent studies (Gerety et al., pers. comm.) further suggest that the NANB-TFA may be retrovirus-like based on the finding of an apparent specific association of reverse transcriptase activity with NANB infectivity. The $CHCl_3$-sensitivity of the NANB-TFA (see below) and the documented propensity of acute disease to progress to chronic infection would appear to lend some support to the above assertion.

PHYSICOCHEMICAL PROPERTIES OF TWO DISTINCT NANB HEPATITIS AGENTS

Although no candidate agent of posttransfusion NANB hepatitis has been serologically linked to the transmission of human disease, several properties of one or more virus-like agents can be inferred from the results of our recent animal transmission

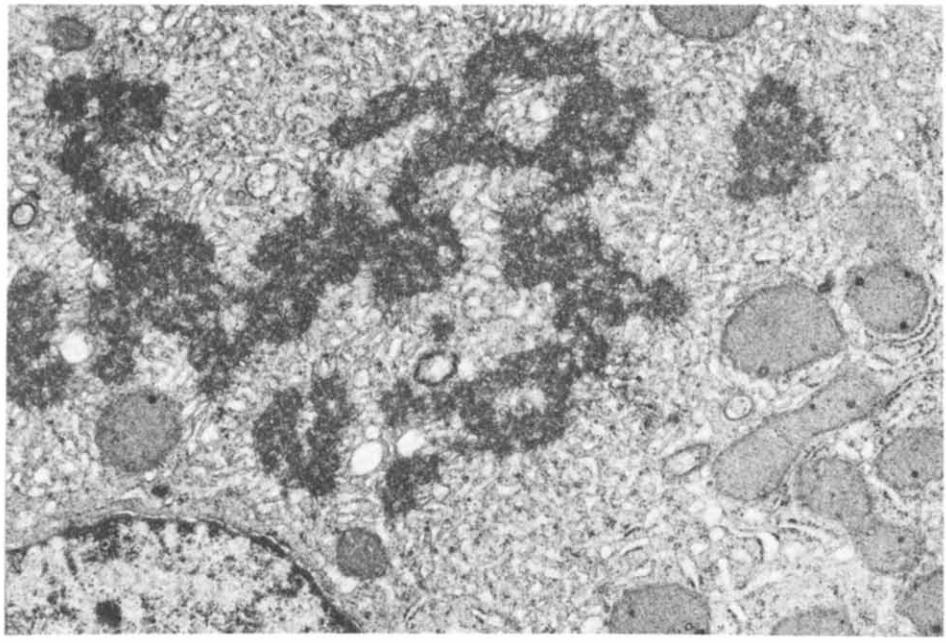

Fig. 4. Dense reticular inclusion body showing extensive involvement of infected chimpanzee hepatocyte cytoplasm (× 15,080).

studies. The agent associated with the induction of hepatocyte cytoplasmic tubules is sensitive to $CHCl_3$ treatment and is most probably an enveloped or lipid-containing virus (Bradley, 1983a, 1984; Feinstone, 1983). The NANB-TFA has also been shown to pass through an 80 nm sharp cut-off polycarbonate membrane filter (Bradley, in press) indicating that it is a relatively small, enveloped (or lipid-containing) virus. The NANB-TFA has recently been recovered from chronic-phase chimpanzee plasma of presumed low titer by use of an extensive purification procedure that assumes the agent is a small, enveloped RNA virus with a buoyant density of 1.24 g/cm^3 in CsCl and a sedimentation coefficient of at least 200 S (Bradley, in press). Studies in our laboratory (Fields, 1983) and elsewhere (Fowler, 1983; Prince, 1982) rule against any similarity of the NANB-TFA to HBV, since nucleic acid hybridization assays, even under conditions of low stringency, have not revealed the presence of a significant sequence homology between these agents. Because HBV (a hepadnavirus) is the only currently known enveloped DNA virus that will pass an 80 nm filter, we have considered the possibility that the NANB-TFA is an enveloped RNA virus. Of the known enveloped RNA viruses, only those belonging to the Togavirus class will readily pass through an 80 nm membrane filter. Although our findings presently suggest the NANB-TFA is Togavirus-like, we cannot exclude the possibility that this agent belongs to another or as yet undefined class of RNA or DNA virus, such as a small retrovirus.

We have also recently presented evidence for the existence of a second agent of NANB-hepatitis that is resistant to $CHCl_3$ treatment (Bradley, 1983a). This agent can be recovered from either chronic phase plasma or acute-phase liver homogenates by a multistep procedure designed for the purification of picornaviruses. This second presumed viral agent of posttransfusion NANB hepatitis does not induce the formation of unique hepatocyte tubules and ERc, however, extensive hepatocyte cytoplasmic vesiculation and vacuolation have been observed in infected chimpanzee liver biopsy specimens during the acute phase of disease. The latter ultrastructural changes are similar in character to those found in cells infected by picornaviruses. These combined findings are in agreement with our previous recovery of 27 nm virus-like particles from a proven-infectious factor VIII concentrate (Bradley, 1979) and from the livers of chimpanzees in the first and second primate passage of the factor VIII-derived NANB hepatitis agent(s) (Maynard and Bradley, 1981). Finally, both NANB hepatitis agents appear to be associated with persistent viremia, and presumably persistent hepatic disease, since chronic-phase plasma from a factor VIII-infected chimpanzee was found to contain both $CHCl_3$-resistant and $CHCl_3$-sensitive agents.

The physicochemical and pathogenetic properties of one major agent of human PTH, the NANB-TFA, are summarized in Tables 1 and 2, respectively.

TABLE 1

Physicochemical properties of a tubule-forming, posttransfusion non-A, non-B hepatitis agent (virus)

(1)	Infectivity is destroyed by:
	(a) formalin 1 : 1,000, 37°C, 96 h
	(b) heat 100°C, 5 min or 60°C, 10 h
	(c) treatment with 20% v/v $CHCl_3$
(2)	Agent contains essential lipid (enveloped)
(3)	Diameter of infectious agent is $\leqslant$80 nm
(4)	Can be pelleted from plasma (assumes agent has an $S_{20,w}$ of $\geqslant$200 S)
(5)	Agent can be recovered from chronic-phase plasma by a multi-step procedure used for the purification of small, enveloped RNA viruses

TABLE 2

Pathogenetic properties of a tubule-forming posttransfusion non-A, non-B hepatitis agent (virus)

(1)	Agent normally causes persistent infection and/or slowly-resolving disease
(2)	Agent interferes with the replication of other hepatotrophic viruses (HAV, HBV)
(3)	Specific ultrastructural changes associated with replication in chimpanzees are confined to hepatocyte cytoplasm
(4)	Ultrastructural changes in chimpanzee hepatocytes are most similar to those induced by some enveloped mammalian RNA viruses
(5)	Titer of virus in majority of inocula reported to be $\leqslant 1 \times 10^3$ CID/ml
(6)	Recrudescence of disease may spontaneously occur >3 yr after the initial infection

SEROLOGIC TESTS AND ALTERNATIVE METHODS

Difficulties encountered in defining the agent (or agents) of human NANB-PTH and in detecting virus-specific antigens and antibodies (Bradley, 1984) probably relate to the low titer of circulating virus and relative deficiency of potent NANB IgG or IgM antibody. In regard to the former issue, most investigators have found that human or chimpanzee inocula generally have titers between 10^2 and 10^4 chimpanzee infectious doses (CID) per ml. For example, our factor VIII agent has a titer of less than 10^3 CID/ml (Bradley, 1983a); the NANB 'F' strain characterized by Feinstone and co-workers (1981) (a chronic phase human plasma) has a titer of less than 10^2 CID/ml; also, a fibrinogen preparation described by Yoshizawa and co-workers (1982) has a titer greater than 10^2 but less than 10^4 CID/ml; an acute phase 'H' strain agent, another plasma described by Feinstone, has a titer of 10^6 CID/ml. Chronic-phase plasma from one of our NANB carrier chimpanzees has recently been shown to have a titer of between 10^4 and 10^5 CID/ml (unpubl. findings) and to cause biochemical and electron microscopic evidence of disease within 4 wk after inoculation into a susceptible animal. Finally, Tabor and Gerety (pers. comm.) have found that one of their NANB inocula has a titer of approximately 10^2 CID/ml. Even if we assume that there are 100 times more defective than infective virions, only the specimen with 10^6 CID/ml may contain detectable antigen, provided a high-titered antibody is used in an optimized immunoassay procedure. If chronic carriers of NANB hepatitis normally have circulating virus titers of less than 10^5 ID/ml, and if excess or soluble virus-specific antigen is not present, then serologic tests of considerably greater sensitivity will be required for the successful detection of particulate viral antigen.

What are the possible alternatives to the development of a conventional serologic test for NANB-PTH? Table 3 lists several newer approaches, many of which are antibody independent in nature or make use of a disease-related phenomenon, such as viral interference. One of the most promising techniques involves the possible detec-

TABLE 3

Alternative test methodologies for the detection of posttransfusion non-A, non-B hepatitis

(1)	Propagation of agent(s) in cell culture with evident CPE or detectable viral interference
(2)	Detection of virus-specific nucleic acid or polypeptides synthesized in cell culture
(3)	Detection of virus- or disease-specific RNA or DNA-dependent polymerase activity in susceptible inoculated cells or patient serum
(4)	Detection of virus- or disease-specific antibody in patient serum or transformed lymphocytes
(5)	Detection of surrogate (non-viral, but disease-specific) antigen in patient serum or peripheral lymphocytes ('turned-on' host gene product)
(6)	Production of cDNA or other hybridization probe suitable for detection of NANB hepatitis-specific nucleic acid in blood

tion of disease-specific antibody produced by transformed lymphocytes (see above). If verified, this procedure could theoretically allow for the identification of NANB-infected blood donors by serologic analysis of in vitro transformed lymphocytes obtained from these individuals.

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