THE JOURNAL OF INFECTIOUS DISEASES • VOL. 148, NO. 2 • AUGUST 1983
 0022-1899/83/4802-0009$01.09

Posttransfusion Non-A, Non-B Hepatitis: Physicochemical Properties of Two Distinct Agents

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Two separate and distinct episodes of non-A, non-B hepatitis were induced in each of two chimpanzees by two inocula: one containing a chloroform-resistant agent and the other containing a chloroform-sensitive agent. Both agents were recovered from liver tissue and plasma obtained from a single chimpanzee during the acute and chronic phases of infection with a factor VIII concentrate, respectively. The chloroform-resistant agent did not cause unique changes in hepatocytes; in contrast, the chloroform-sensitive agent did induce the formation of cytoplasmic tubules, convoluted endoplasmic reticulum, and dense reticular inclusion bodies. The latter changes are similar in character to those induced in infected cells by some enveloped mammalian RNA viruses.

Recent studies have demonstrated that posttransfusion non-A, non-B (NANB) hepatitis of human origin can be transmitted to chimpanzees by iv or percutaneous inoculation of infected plasma, serum, factor VIII concentrates, factor IX preparations, or commercially prepared fibrinogen [1–6]. The occurrence of multiple episodes of NANB hepatitis in some individuals [7, 8] and the variation in the length of the incubation period for this disease, both in patients [9–13] and in experimentally infected chimpanzees [1–6], have been cited as presumptive evidence for the existence of two or more etiologic agents. Cross-challenge studies in experimentally infected chimpanzees [14–16] have also provided substantial support for the notion that more than one agent is responsible. This report documents the induction of two separate and distinct episodes of NANB hepatitis in each of two chimpanzees by two defined inocula: one containing a chloroform-resistant agent and the other containing a chloroform-sensitive agent.

Received for publication December 1, 1982, and in revised form March 24, 1983.

We thank Dr Stephen Feinstone for his helpful discussion and comments.

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Materials and Methods

Chimpanzees. Colony-born chimpanzees 1030, 1041, and 1091 were housed and instrumented as previously described [3]. The sera of chimpanzees 1030 and 1041 contained antibody to hepatitis B surface antigen but not that to hepatitis A virus. The serum of chimpanzee 1091 contained neither of these antibodies.

Inoculum I. An aliquot of one lot of a commercial antihemophilic material implicated in the transmission of NANB hepatitis to a patient and subsequently shown to transmit NANB hepatitis to chimpanzees [3] was used for the preparation of inoculum I. Reconstituted factor VIII was diluted 1:1,000 in normal chimpanzee serum.

Inoculum II. A challenge inoculum was prepared by the combination of three plasma specimens from a chimpanzee that was a proved carrier of NANB hepatitis (animal 771) [17, 18]. These plasma samples were obtained 45–48 months after inoculation of 30 ml of the undiluted factor VIII materials used for the preparation of inoculum I. Inoculum II was prepared from the plasma pool as follows. First, 6.0 ml of plasma was diluted to a volume of 38.5 ml in 0.01 *M* PBS (pH 7.4) and pelleted in a Beckman SW27 rotor (Beckman Instruments, Fullerton, Calif) at 120,000 *g* for 5 hr at 20 C. The supernatant was then decanted, and

the pellet was resuspended in 10.0 ml of PBS and vortexed at room temperature (~25 C) for 10 min prior to inoculation. This pelleting procedure involves the assumption that the infectious agent is particulate and has a sedimentation coefficient of at least 157S.

Inoculum III. Liver tissue was obtained from chimpanzee 771 during the acute phase of NANB hepatitis. Cesium chloride density-gradient fractions of crude liver homogenates prepared from this tissue had previously been shown to cause NANB hepatitis in iv-inoculated chimpanzees [3, 19]. For our study, aliquots of the same liver tissue were subjected to a more extensive purification procedure than had been used before. In this procedure, 1.0 g of liver tissue was immersed in 20.0 ml of ice-cold PBS (pH 7.4), minced with scissors, and homogenized in a Sorval Omnimixer (Ivan Sorval, Norwalk, Conn) at top speed. The homogenate was filtered through four layers of cheesecloth and clarified by centrifugation at 12,000 *g* for 20 min at 25 C. Next, 4.0 ml of the supernatant solution was extracted with five volumes (20 ml) of chloroform (83%, vol/vol) by vortexing of the mixture for 10 min at 25 C. The suspension was separated by centrifugation at 775 *g* for 15 min at 25 C. The aqueous phase was removed, diluted 10-fold in 0.01 *M* Tris buffer (pH 7.4) and 0.001 *M* EDTA (Tris-EDTA), and centrifuged in a Beckman Ti-60 rotor at 260,000 *g* for 2 hr at 5 C. The resulting viral pellets were resuspended in 2 ml of Tris-EDTA plus 0.5% bovine serum albumin (BSA). The suspension was layered onto a preformed cesium chloride density gradient (1.174–1.500 g/cm^3) and centrifuged in a Beckman SW27 rotor at 105,000 *g* for 20 hr at 5 C. Thirty-two fractions of 1.2 ml each were collected in siliconized tubes containing 0.5% (wt/vol) BSA. Fractions with buoyant densities of 1.30–1.36 g/cm^3 were pooled, diluted sixfold in Tris-EDTA plus 0.5% BSA, and centrifuged in an SW27 rotor at 110,000 *g* for 5.0 hr at 5 C for the recovery of virus. The pellets were resuspended in 0.01 *M* Tris buffer (pH 7.4) containing 0.5% BSA. This purification procedure is used on the assumption that the infectious agent is insensitive to lipid solvents, has a sedimentation coefficient of ⩾157S, and possesses a buoyant density of 1.30–1.36 g/cm^3.

Inoculum IV. Inoculum IV was prepared in exactly the same manner as inoculum III except that chloroform extraction of the clarified crude liver homogenate was omitted.

Inoculum V. Inoculum V was prepared in exactly the same manner as inoculum II except that

Table 1. Description of inocula used in NANB hepatitis infectivity studies in chimpanzees.

Inoculum	Source [reference(s)]	Method of preparation	Infectivity and ultrastructural changes in chimpanzees
I	Proved-infectious factor VIII concentrate [3]	Diluted 1:1,000 in normal chimpanzee serum	Noninfectious (control): no ALT elevation or electron microscopic changes; normal liver histology
II	Chronic-phase plasma from chimpanzee 771, a proved NANB hepatitis carrier [17, 18]	Pelleted at 120,000 *g* for 5 hr; pellet resuspended in PBS	Induced NANB hepatitis: elevated ALT activity; cytoplasmic vesiculation in hepatocytes, with tubules and convoluted endoplasmic reticulum; abnormal liver histology
III	Proved-infectious acute-phase liver homogenate from chimpanzee 771 [3, 19]	Homogenate pelleted; pellet treated with 83% $CHCl_3$ and banded on CsCl gradient; gradient fractions of 1.30–1.36 g/cm^3 pooled, diluted, and pelleted; pellet resuspended in Tris-BSA	Induced NANB hepatitis: elevated ALT activity; severely abnormal liver histology; cytoplasmic vesiculation and vacuolation in hepatocytes; no tubules or convoluted endoplasmic reticulum present
IV	Same as inoculum III	Same as for inoculum III except for omission of $CHCl_3$ treatment	Noninfectious: no ALT elevation during 10 weeks of observation
V	Same as inoculum II	Same as for inoculum II, but resuspended pellet treated with 20% $CHCl_3$	Induced NANB hepatitis: elevated ALT activity; cytoplasmic vesiculation in hepatocytes; no tubules or convoluted endoplasmic reticulum

2.5 ml of chloroform (20%, vol/vol) was added to the 10.0-ml suspension prior to vortexing.

Chloroform extraction control studies. (1) plasma. Recovery of typical unenveloped and enveloped viruses from normal chimpanzee plasma subjected to the purification scheme used for inoculum II was accomplished via the seeding of whole plasma and plasma pellets with either 1.0 ml of poliovirus (titer, 10^6 infectious doses [ID]/ml) or 1.0 ml of bovine parainfluenza virus (titer, 10^6 ID/ml) as follows. Two 6.0-ml aliquots of plasma, one of which was seeded with virus, were diluted in PBS and pelleted as already described. The pellet containing virus was resuspended in 10.0 ml of PBS, and the pellet free of virus was resuspended in 9.0 ml of PBS and seeded with 1.0 ml of virus. Chloroform (2.5 ml) was added to each suspension prior to vortexing. Titers of virus were determined in resuspended plasma pellets prior to and after chloroform extraction by the end-point dilution method; the Vero monkey kidney and bovine embryonic kidney cell lines were used for poliovirus and bovine parainfluenza virus, respectively.

(2) Liver. Recovery of two control viruses from a normal chimpanzee liver homogenate subjected to the purification scheme used in the preparation of inoculum III was accomplished by the seeding of 9.0-ml aliquots of clarified liver homogenate with either 1.0 ml of poliovirus (titer, 10^7 ID/ml) or 1.0 ml of bovine parainfluenza virus (titer, 10^7 ID/ml). Seeded liver homogenates were purified as described for inoculum III, and the final suspensions were assayed for remaining virus. The titer of virus remaining at several intermediate stages of purification was also determined.

Serologic tests. Hepatitis B surface antigen, antibody to hepatitis B surface antigen, antibody to hepatitis B core antigen, and antibody to hepatitis A virus in serial serum specimens were routinely determined by Ausria II, Ausab, CORAB, and HAVAB (Abbott Laboratories, North Chicago, Ill), respectively. Titers of antibody to cytomegalovirus (CMV) and Epstein-Barr virus (EBV) were determined as previously described [3].

Blood samples were obtained twice weekly for the determination of serum alanine aminotransferase (ALT) activity. This activity was measured by a manual colorimetric procedure, as previously described [3]. A cutoff value representing the 99% confidence limit for the upper level of normal ALT activity in a given chimpanzee was established by calculation of the mean of 16 values obtained before inoculation and addition of 2.95 standard deviations of the mean to this value.

Electron microscopy. Liver biopsy specimens were obtained weekly by Menghini needle aspiration. Liver tissue was fixed and stored until use at 4 C in 4% formaldehyde (prepared from paraformaldehyde) in 0.1 *M* phosphate buffer (pH 7.2). Samples for electron microscopy were postfixed in osmic acid, stained with 1% uranyl acetate, dehydrated in a graded series of ethanols, and embedded in Epon araldite (Polysciences, Warington, Pa). Thin sections were stained with uranyl acetate and lead citrate.

Light microscopy. Serial (weekly) liver biopsy specimens obtained from chimpanzees 1030 and 1041 during their first episodes of NANB hepatitis were examined by light microscopy for histologic evidence of acute viral hepatitis.

Results

Control studies in chimpanzee 1091. Intravenous inoculation of chimpanzee 1091 with a 1:1,000 dilution of one lot of infectious factor VIII materials (inoculum I; table 1) [3] did not result in elevated ALT activity during 28 weeks of observation (figure 1). Enzyme activity remained well within the range of the 16 ALT values obtained before inoculation and never exceeded the 99% confidence limit for normal ALT activity. Challenge of this animal with inoculum II, however, did cause significant elevations in ALT activity, which peaked at 70 milliunits/ml 10 weeks after inoculation. There was no serologic evidence of acute infection or reinfection with hepatitis A virus (HAV), hepatitis B virus (HBV), CMV, or EBV during the episode of NANB hepatitis in animal 1091.

Electron microscopic examination of serial liver biopsy specimens from animal 1091 revealed no abnormalities in hepatocyte ultrastructure after iv administration of inoculum I. Unique NANB hepatitis-associated ultrastructural changes, however, were found in the cytoplasm of hepatocytes in liver biopsy specimens obtained two weeks after infusion of inoculum II. These changes consisted of the commonly described tubules [5, 14, 15, 20,

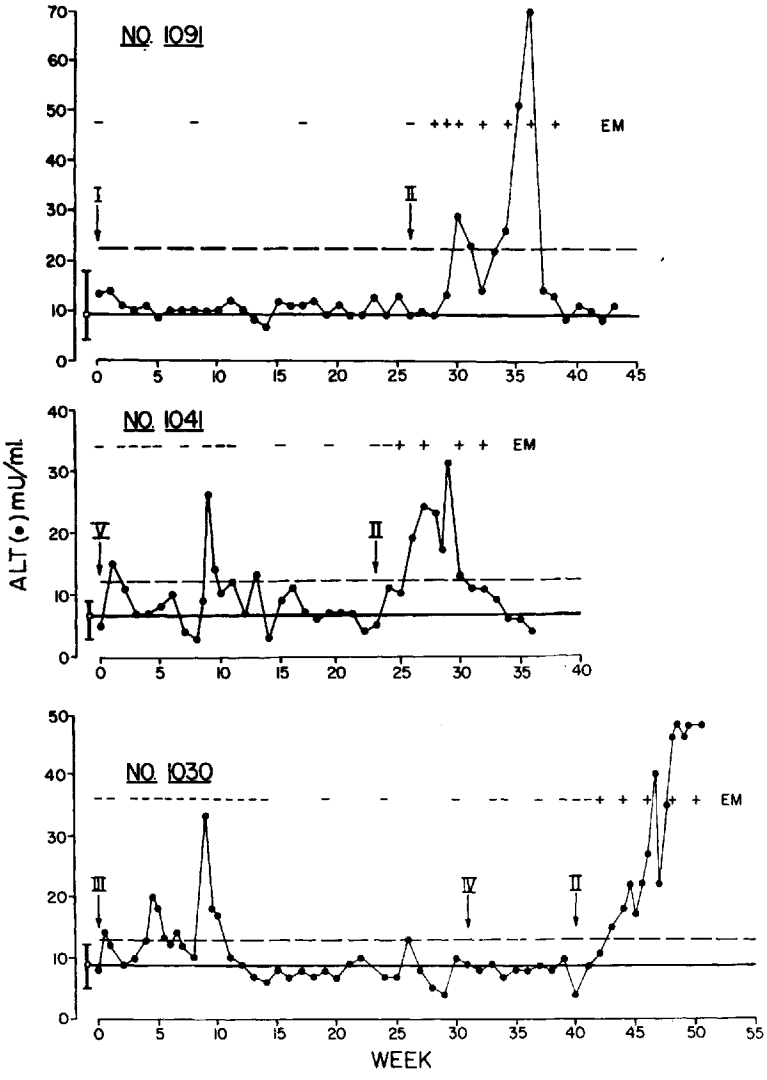

Figure 1. ALT activity in sera of chimpanzees inoculated with factor VIII materials and/or partially purified preparations of acute-phase liver and chronic-phase plasma containing chloroform-resistant and chloroform-sensitive agents of NANB hepatitis. Roman numerals refer to specific inocula administered at the indicated week. The vertical bar in each panel represents the range of 16 ALT values obtained before inoculation, with the mean value indicated by an open circle. The dashed horizontal line in each panel represents a statistically determined cutoff value equivalent to the 99% confidence limit for the upper level of normal ALT activity in that animal [14, 17, 18]. EM = electron microscopy; (+) = tubules present; (−) = tubules absent; mU = milliunits.

21] as well as numerous convoluted bilayer membranes derived from proliferated endoplasmic reticulum [21–23]. Dense reticular inclusion bodies [21, 22] were also found in the cytoplasm of infected hepatocytes during the peak in the elevation of ALT activity. The latter structures are morphologically similar to the viroplasmic foci ("virus factories") previously identified in cells infected by some enveloped mammalian RNA viruses, including murine coronavirus [24], flavivirus [25, 26], and influenza virus [27].

Chloroform extraction control studies. (1) Plasma. The recovery of poliovirus from seeded whole plasma and a seeded plasma pellet was unaffected by chloroform extraction or pelleting; no loss of infectivity was observed after either treatment. Pelleting of bovine parainfluenza virus from seeded whole plasma, however, resulted in a moderate loss of infectivity (10^2 ID/ml). Treatment of pelleted virus and a seeded plasma pellet with 20% (vol/vol) chloroform resulted in the complete loss of infectivity of bovine parainfluenza virus in both preparations.

(2) Liver. The recovery of poliovirus from seeded liver homogenates was moderately affected by the extensive purification scheme used in the preparation of inoculum III. Approximately 10^2 ID of poliovirus infectivity/ml was lost during the purification procedure. The loss was not due to extraction with 83% (vol/vol) chloroform, since 10^5 ID of virus/ml was recovered after an identical extraction procedure that did not involve the use of chloroform. The infectivity of bovine parainfluenza virus in seeded liver preparations, how-

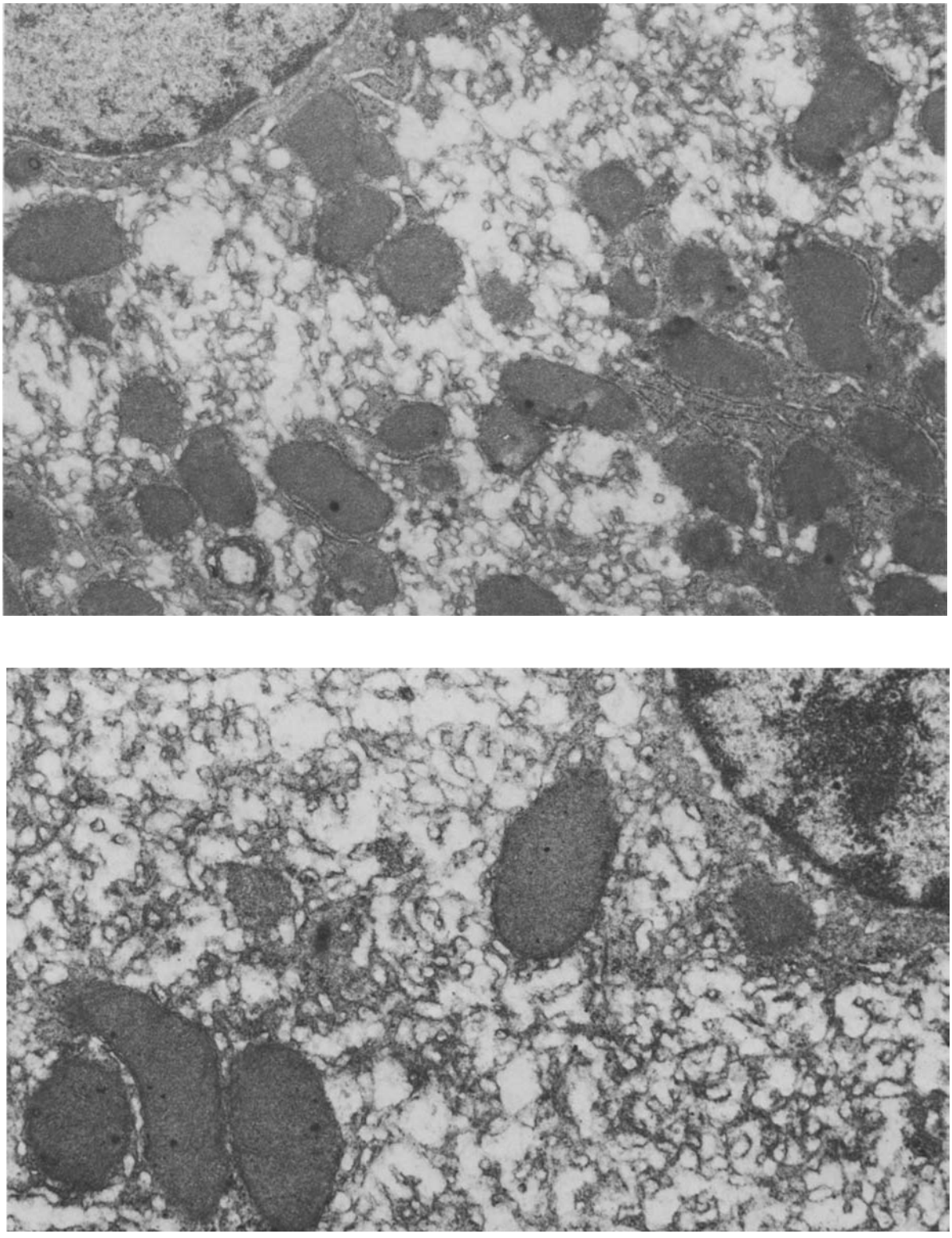

Figure 2. Cytoplasmic vacuolation and vesiculation in hepatocytes in a liver biopsy specimen obtained from chimpanzee 1041 28 days after administration of inoculum V. The endoplasmic reticulum is hypertrophied and in places forms numerous small vesicles (top, ×17,600; bottom, ×25,800).

ever, was completely lost after the initial extraction with 83% (vol/vol) chloroform.

Transmission of infection by chronic-phase plasma fractions. Intravenous inoculation of chimpanzee 1041 with a chloroform-treated, chronic-phase plasma fraction (inoculum V) resulted in elevated ALT activity approximately one week later (figure 1). ALT activity reached a maximal level nine weeks after inoculation and returned to base-line values nine weeks later. Reinoculation of animal 1041 with an identical plasma fraction that had not been subjected to chloroform extraction (inoculum II) induced a second episode of NANB hepatitis after an incubation period of three weeks. No serologic evidence of acute infection or reinfection with HAV, HBV, CMV, or EBV was found during either episode of hepatitis. Light-microscopic examination of liver biopsy specimens obtained from this animal during the first episode of hepatitis revealed the presence of parenchymal lesions that were consistent with a diagnosis of acute viral hepatitis [3]. Examination of the same liver biopsy specimens by electron microscopy revealed extensive cytoplasmic vesiculation and abnormal hypertrophy of the endoplasmic reticulum that were coincident with the presence of severe parenchymal lesions (figure 2). No convoluted endoplasmic reticulum, tubules, or dense reticular inclusion bodies were observed in any of 10 serial liver biopsy specimens obtained during the major period of elevated ALT activity. Electron microscopic examination of liver biopsy specimens obtained during the second episode of disease, however, showed the presence of uniquely convoluted endoplasmic reticulum, tubules, and dense reticular inclusion bodies in the cytoplasm of hepatocytes (figure 3).

Transmission of infection by purified acute-phase liver preparations. Intravenous inoculation of chimpanzee 1030 with inoculum III (table 1) resulted in significant elevations of enzyme activity within one week; major peaks of ALT activity occurred approximately four and nine weeks after inoculation (figure 1). Subsequent challenge of this animal with an identical liver preparation that had not been subjected to chloroform extraction (inoculum IV) failed to induce a second instance of elevated ALT activity (figure 1). However, a second challenge inoculum, consisting of pelleted chronic-phase plasma (inoculum II) and administered 40 weeks after the first inoculation, did cause significant enzyme elevations three weeks after iv infusion (figure 1). No serologic evidence of acute infection or reinfection with HAV, HBV, CMV, or EBV was found during either episode of hepatitis.

Examination of serial liver biopsy specimens from animal 1030 by light microscopy revealed only mild sinusoidal activation prior to administration of inoculum III; however, these changes became more obvious immediately after inoculation with this preparation (figure 4). The activation of sinusoidal cells was conspicuous on day 28 but even more so on day 35, when the first hepatocellular alterations (some cytoplasmic clumping and mild steatosis), coincident with elevations in ALT activity, were noted. The latter changes were also present on day 43, during the time when the most severe histologic changes were seen (figure 4). Some focal necroses were also present on day 43. In this specimen, many portal tracts were noted; they were expanded and contained a large number of mainly mononuclear inflammatory cells that, in places, extended into the surrounding parenchyma, with a minor loss of hepatocytes in a mild, piecemeal necrosis. The bile ductules were proliferated, and inflammatory cells were noted within the epithelium of bile ducts. In subsequent specimens both lobular and portal tract lesions were present but were far less severe, with the portal tract lesions showing a tendency to a mild degree of fibrosis. Thus, the major histologic insult on day 43 apparently preceded the major peak of ALT activity during the first episode of NANB hepatitis in chimpanzee 1030.

Electron microscopic examination of serial liver biopsy specimens obtained from chimpanzee 1030 during the first episode of NANB hepatitis revealed abnormal cytoplasmic vesiculation in hepatocytes, with hypertrophy of the smooth endoplasmic reticulum as early as 11 days after inoculation. Cytoplasmic vesiculation was most prominent 43 days after inoculation and coincided with the appearance of the most severe histologic lesions (figure 5). One of the most notable ultrastructural changes was the appearance of numerous, highly convoluted lamellae within the Golgi complex 28 days after inoculation (figure 5, upper left). No tubules, convoluted endoplasmic reticulum, or dense reticular inclusion bodies were visualized in any biopsy specimen obtained during the first 40 weeks of study (figure 1). Crystalline

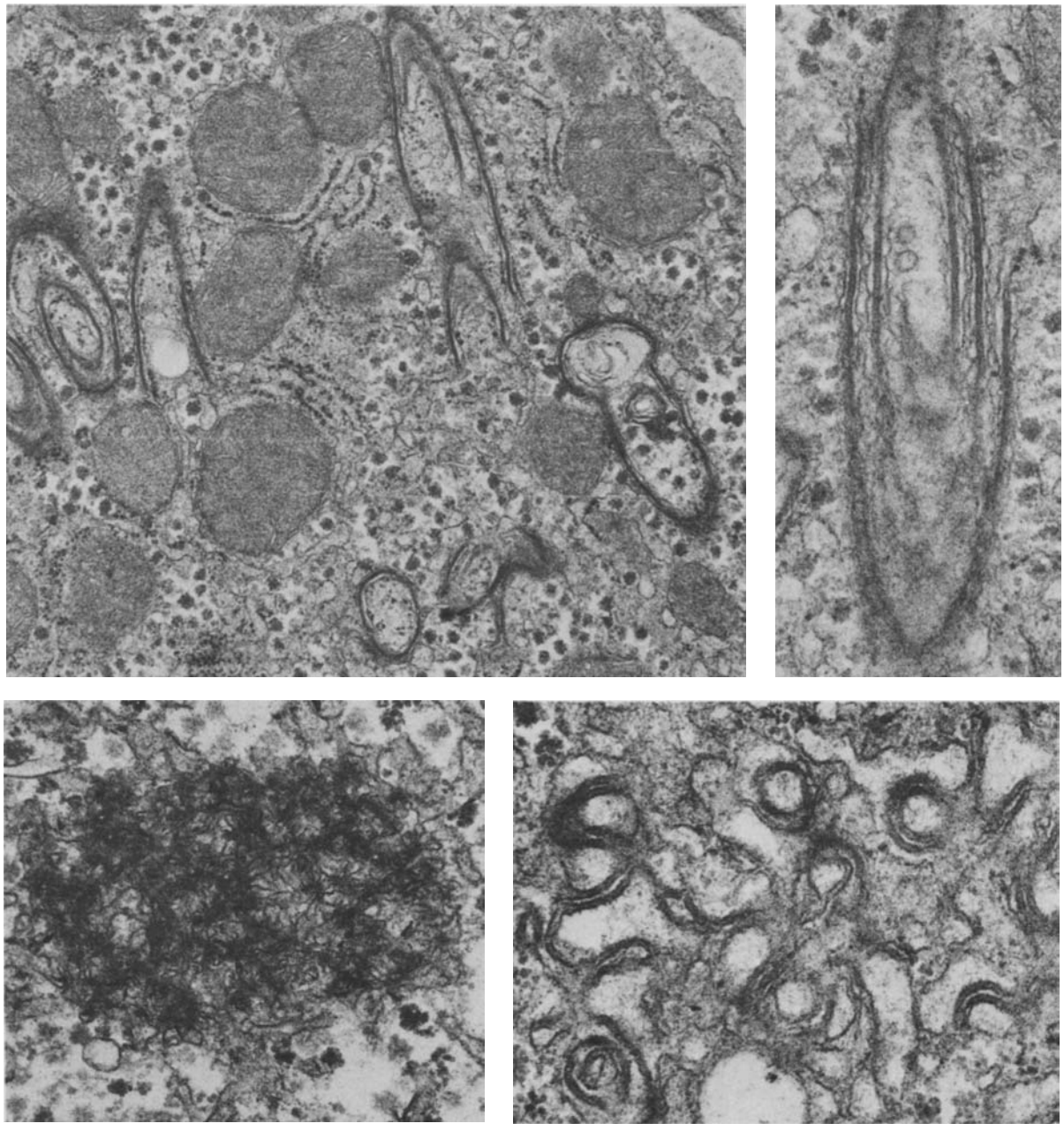

Figure 3. Typical ultrastructural alterations in the cytoplasm of hepatocytes obtained from chimpanzee 1041 during infection with a chloroform-sensitive agent of NANB hepatitis (inoculum II). Upper left: Tubules derived from proliferated endoplasmic reticulum (×26,120). Upper right: Multilaminated tubular structure showing dense, osmiophilic substance bounded by single-unit membranes derived from proliferated endoplasmic reticulum (×52,260). Lower left: Dense reticular inclusion body with numerous foci and radiating strandlike filaments, some of which are contiguous with the surrounding endoplasmic reticulum (×52,260). Lower right: convoluted endoplasmic reticulum with osmiophilic substance entrapped between paired cisternae (×52,260).

arrays of electron-dense particles were also observed in endothelial cells of specimens obtained 28 and 43 days after inoculation (figure 5, bottom). Nuclear changes were most evident on day 43 and consisted of crenellated nuclear membranes and clumped and marginated chromatin. Clusters of densely stained 15- to 25-nm granules were also visualized in a limited number of hepatocyte nuclei (figure 5, upper right).

Challenge of chimpanzee 1030 with inoculum

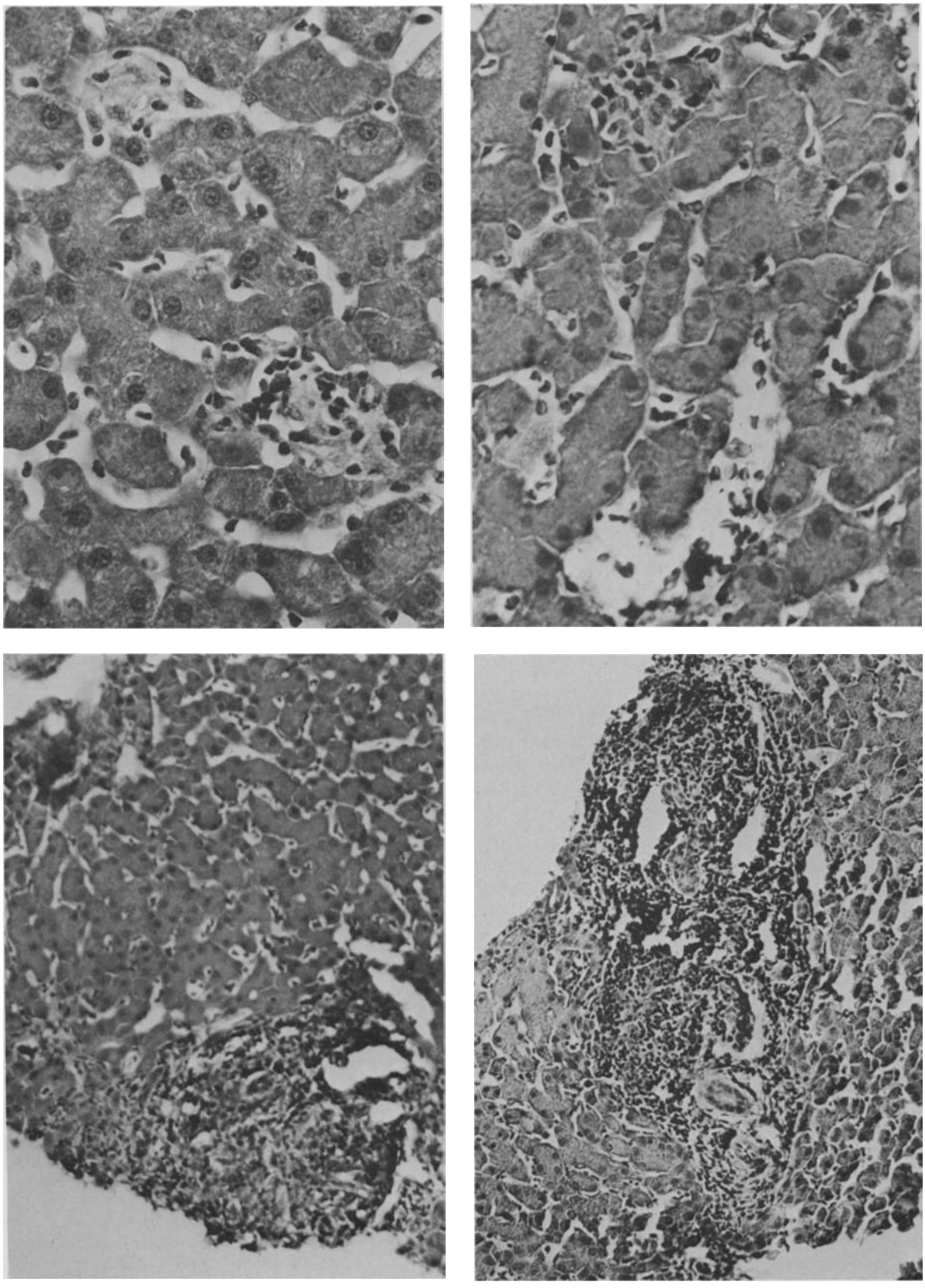

Figure 4. Liver histology in chimpanzee 1030 after infection with inoculum III. Upper left, day 11: Mild increase in numbers of sinusoidal cells, which have accumulated focally in a conspicuous manner. The hepatocyte cytoplasm appears normal (hematoxylin and eosin; ×240). Upper right, day 43: The cytoplasm of the hepatocytes appears, in places, to be irregularly clumped; the number of sinusoidal cells is conspicuously increased, and in some areas

IV did not result in ultrastructural changes in hepatocytes. However, two weeks after challenge with inoculum II, convoluted endoplasmic reticulum and tubules were observed in the cytoplasm of hepatocytes (figure 1). Subsequent specimens contained dense reticular inclusion bodies in addition to the more abundant tubules and convoluted endoplasmic reticulum.

Discussion

We have previously reported the transmission of NANB hepatitis to chimpanzees via factor VIII materials, crude liver fractions, and acute- and chronic-phase plasma specimens [3, 14, 17–19]. In nearly every case, unique tubular structures derived from proliferated endoplasmic reticulum were visualized in the cytoplasm of infected hepatocytes [14, 23]. However, several discrepancies in the temporal patterns of ultrastructural changes in liver biopsy specimens were noted. Some animals that received the same inoculum developed similar profiles of elevated ALT activity but did not demonstrate the expected concordance in electron microscopic changes [23]. Furthermore, cross-challenge studies conducted in our laboratory showed that some episodes of NANB hepatitis in chimpanzees were not associated with the appearance of tubules or convoluted endoplasmic reticulum [14].

The findings of our current study suggest that two etiologic agents are responsible for the transmission of posttransfusion NANB hepatitis. These agents are physicochemically distinct and can be differentiated on the basis of their sensitivity to chloroform treatment. This conclusion is based in part on the assumption that the chimpanzees used in these studies did not have latent viral infections that might have been reactivated by infusion of any of the experimental inocula. In fact, previous studies in our laboratory have shown that chimpanzees given an iv inoculation of noninfectious plasma from chimpanzees (up to 100 ml), homogenates of liver tissue from marmosets or chimpanzees, or suspensions of stool from chimpanzees do not exhibit enzymatic or histologic evidence of nonspecific or spontaneous liver disease (authors' unpublished data).

The first episode of NANB hepatitis in chimpanzees 1030 and 1041 was induced by infection with a chloroform-resistant agent that was pelleted from a partially purified liver preparation in one instance and a chronic-phase plasma pool in the other. In animal 1030, the infectivity of a liver preparation from a chimpanzee with acute NANB hepatitis was demonstrated after an extensive purification procedure that was specifically designed to recover viruslike particles with properties similar to those of picornaviruses or small, unenveloped RNA viruses. Of interest is that the cytoplasmic vesiculation visualized in hepatocytes in some biopsy specimens obtained from animal 1030 during the first episode of NANB hepatitis was morphologically similar to that observed in cultured cells infected by mammalian picornaviruses [28–30]. This finding is consistent with our earlier-reported recovery of 27-nm viruslike particles from the factor VIII preparation and cesium chloride gradient fractions of the liver homogenate from chimpanzee 771 [3].

Challenge of animal 1030 with inoculum IV failed to induce biochemical, histologic, or electron microscopic evidence of NANB hepatitis. Although we have previously demonstrated that the transmission of short-incubation NANB hepatitis (associated with the formation of tubules) to chimpanzees via cesium chloride gradient-fraction pools of an isopycnically banded crude liver homogenate from animal 771 [3, 19], additional purification of the acute-phase liver homogenate obviously resulted in a complete loss of infectivity of the tubule-forming agent. Rechallenge of animal 1030 with inoculum II, however, caused a second episode of NANB hepatitis that was accompanied by the appearance of tubules, convoluted endoplasmic reticulum, and dense reticular inclusion bodies. As in chimpanzee 1030, challenge of animal 1041 with inoculum II resulted in renewed elevations of enzyme activity and the appearance of tubules, convoluted endoplasmic

these cells have replaced hepatocytes, which have disappeared (focal necrosis) (hematoxylin and eosin, ×240). Lower left, day 43: Conspicuous enlargement of densely infiltrated portal tract. In places, inflammatory cells have replaced periportal hepatocytes. The number of sinusoidal cells has increased conspicuously (hematoxylin and eosin, ×100). Lower right, day 43: Conspicuous enlargement of densely infiltrated portal tract, with focal extension of inflammatory cells into the surrounding parenchyma, where hepatocytes have been lost. Note infiltration of epithelium of bile duct in center (hematoxylin and eosin, ×100).

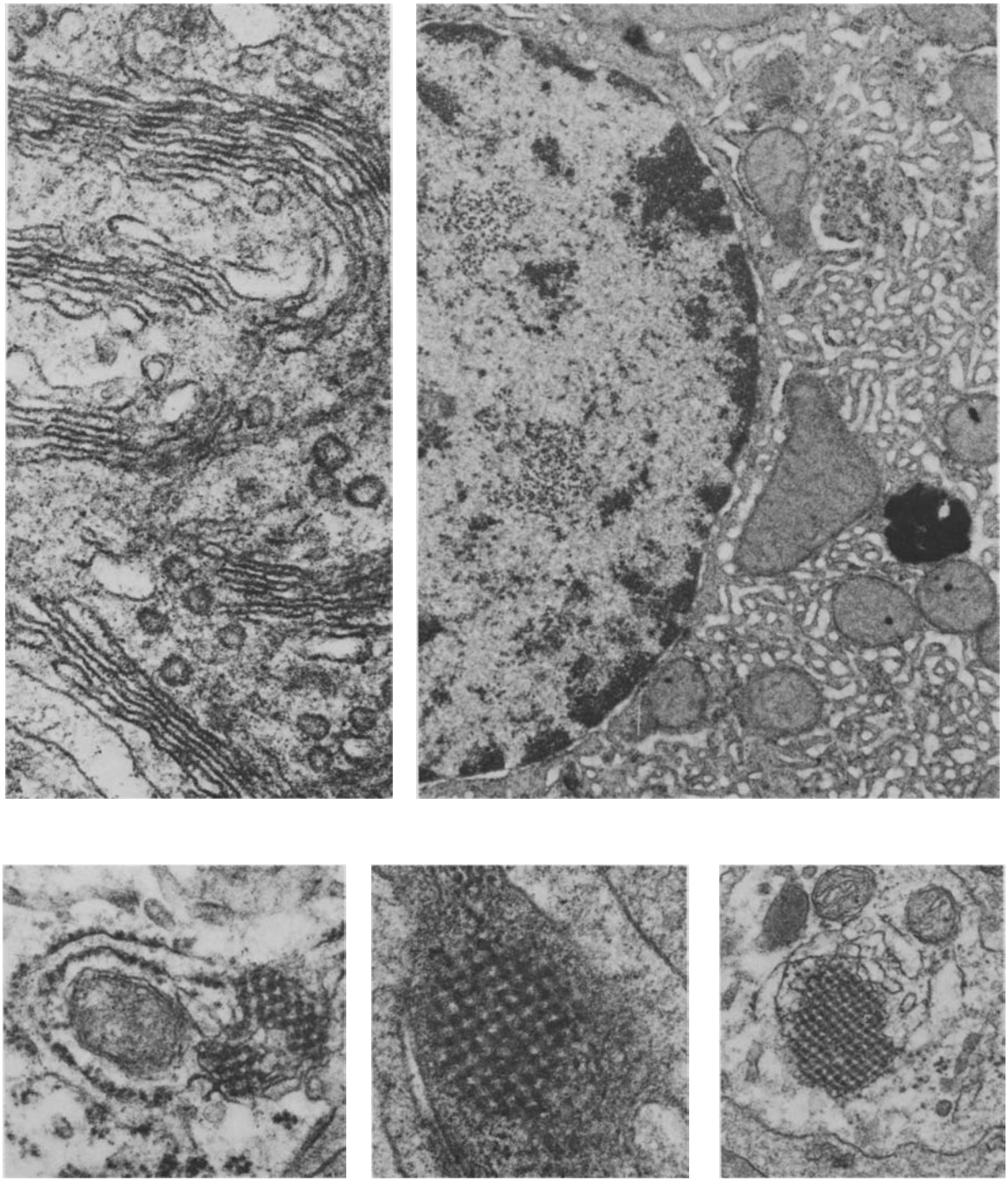

Figure 5. Cytoplasmic changes in hepatocytes in liver biopsy specimens obtained from chimpanzee 1030 during its first episode of NANB hepatitis (after infection with inoculum III). Upper left, day 28: Highly convoluted membranes within a Golgi complex in a hepatocyte (×85,600). Upper right, day 43: Cytoplasmic vesiculation in a hepatocyte, with rounding and swelling of mitochondria. Clusters of dense granules are present in the nucleus (×17,600). Bottom, days 28 and 43: Crystalline arrays of 25- to 30-nm, densely stained particles in endothelial cells. These arrays were sometimes associated with (or connected to) membranous structures (far left, ×52,300; middle, ×79,300; far right, ×40,000).

reticulum, and dense reticular inclusion bodies in hepatocyte cytoplasm. This finding reinforces the notion that two distinct agents are present in the factor VIII inoculum and that both may be associated with persistent viremia.

In summary, our findings suggest that (*1*) there are at least two distinct agents of posttransfusion NANB hepatitis; (*2*) the chloroform-sensitive agent is associated with the formation of cytoplasmic tubules, convoluted endoplasmic reticulum, and dense reticular inclusion bodies in hepatocytes; (*3*) the titer of neither agent was $\geq 1 \times 10^3$ chimpanzee ID/ml in the original factor VIII inoculum; and (*4*) both agents of NANB hepatitis are associated with ultrastructural alterations in hepatocyte cytoplasm.

References

1. Alter HJ, Purcell RH, Holland PV, Popper H. Transmissible agent in non-A, non-B hepatitis. Lancet 1978;**1**: 459–63
2. Tabor E, Gerety RJ, Drucker JA, Seeff LB, Hoofnagle JH, Jackson DR, April M, Barker LF, Pineda-Tamondong G. Transmission of non-A, non-B hepatitis from man to chimpanzee. Lancet 1978;**1**:463–6
3. Bradley DW, Cook EH, Maynard JE, McCaustland KA, Ebert JW, Dolana GH, Petzel RA, Kantor RJ, Heilbrunn A, Fields HA, Murphy BL. Experimental infection of chimpanzees with antihemophilic (factor VIII) materials: recovery of virus-like particles associated with non-A, non-B hepatitis. J Med Virol 1979;**3**:253–69
4. Hollinger FB, Gitnick GL, Aach RD, Szmuness W, Mosley JW, Stevens CE, Peters RL, Weiner JM, Werch JB, Lander JJ. Non-A, non-B hepatitis transmission in chimpanzees: a project of the transfusion transmitted viruses study group. Intervirology 1978;**10**:60–8
5. Yoshizawa H, Akahane Y, Itoh Y, Iwakiri S, Kitajima K, Morita M, Tanaka A, Nojiri T, Shimizu M, Miyakawa Y, Mayumi M. Viruslike particles in a plasma fraction (fibrinogen) and in the circulation of apparently healthy blood donors capable of inducing non-A/non-B hepatitis in humans and chimpanzees. Gastroenterology 1980;**79**:512–20
6. Wyke RJ, Tsiquaye KN, Thornton A, White Y, Portmann B, Das PK, Zuckerman AJ, Williams R. Transmission of non-A, non-B hepatitis to chimpanzees by factor-IX concentrates after fatal complications in patients with chronic liver disease. Lancet 1979;**1**:520–4
7. Mosley JW, Redeker AG, Feinstone SM, Purcell RH. Multiple hepatitis viruses in multiple attacks of acute viral hepatitis. N Engl J Med 1977;**296**:75–8
8. Hruby MA, Schauf V. Transfusion-related short-incubation hepatitis in hemophilic patients. JAMA 1978;**240**: 1355–7
9. Aach RD, Lander JJ, Sherman LA, Miller WV, Kahn RA, Gitnick GL, Hollinger FB, Werch J, Szmuness W, Stevens CE, Kellner A, Weiner JM, Mosely JW. Transfusion-transmitted viruses: interim analysis of hepatitis among transfused and nontransfused patients. In: Vyas GN, Cohen SH, Schmid R, eds. Viral hepatitis: a contemporary assessment of etiology, epidemiology, pathogenesis, and prevention. Philadelphia: Franklin Institute Press, 1978:383–96
10. Craske J, Dilling N, Stern D. An outbreak of hepatitis associated with intravenous injection of factor-VIII concentrate. Lancet 1975;**2**:221–3
11. Ahtone J, Francis D, Bradley D, Maynard J. Non-A, non-B hepatitis in a nurse after percutaneous needle exposure. Lancet 1980;**1**:1142
12. Guyer B, Bradley DW, Bryan J, Maynard JE. Non-A, non-B hepatitis among participants in a plasmapheresis stimulation program. J Infect Dis 1979;**139**:634–40
13. Tateda A, Kikuchi K, Numazaki Y, Shirachi R, Ishida N. Non-A, non-B hepatitis in Japanese recipients of blood transfusions: clinical and serologic studies after the introduction of laboratory screening of donor blood for hepatitis B surface antigen. J Infect Dis 1979;**139**:511–8
14. Bradley DW, Maynard JE, Cook EH, Ebert JW, Gravelle CR, Tsiquaye KN, Kessler H, Zuckerman AJ, Miller MF, Ling C, Overby LR. Non-A/non-B hepatitis in experimentally infected chimpanzees: cross-challenge and electron microscopic studies. J Med Virol 1980;**6**:185–201
15. Yoshizawa H, Itoh Y, Iwakiri S, Kitajima K, Tanaka A, Nojiri T, Miyakawa Y, Mayumi M. Demonstration of two different types of non-A, non-B hepatitis by reinjection and cross-challenge studies in chimpanzees. Gastroenterology 1981;**81**:107–13
16. Hollinger FB, Mosley JW, Szmuness W, Aach RD, Peters RL, Stevens C. Transfusion-transmitted viruses study: experimental evidence for two non-A, non-B hepatitis agents. J Infect Dis 1980;**142**:400–7
17. Bradley DW, Maynard JE, Popper H, Ebert JW, Cook EH, Fields HA, Kemler BJ. Persistent non-A, non-B hepatitis in experimentally infected chimpanzees. J Infect Dis 1981;**143**:210–8
18. Bradley DW, Maynard JE, Krawczynski KZ, Popper H, Cook EH, Gravelle, CR, Ebert JW. Non-A/non-B hepatitis in chimpanzees infected with a factor VIII agent: evidence of persistent hepatic disease. In: Alter HJ, Maynard JE, Szmuness W, eds. Viral hepatitis. Proceedings of the 1981 International Symposium on Viral Hepatitis. Philadelphia: Franklin Institute Press, 1982:319–29
19. Maynard JE, Bradley DW. Transmission of non-A, non-B hepatitis by blood products and plasma derivatives. In: Gerety RJ, ed. Non-A, non-B hepatitis. New York: Academic Press, 1981:71–95
20. Shimizu YK, Feinstone SM, Purcell RH, Alter HJ, London WT. Non-A, non-B hepatitis: ultrastructural evidence for two agents in experimentally infected chimpanzees. Science 1979;**205**:197–200
21. Pfeifer U, Thomssen R, Legler K, Bottcher U, Gerlich W, Weinmann E, Klinge O. Experimental non-A, non-B hepatitis: four types of cytoplasmic alteration in hepatocytes of infected chimpanzees. Virchows Arch [Cell Pathol] 1980;**33**:233–43

22. Bradley DW, Krawczynski KZ, Cook EH, Gravelle CR, Ebert JW, Maynard JE. Recrudescence of non-A, non-B hepatitis in persistently infected chimpanzees. In: Viral hepatitis—proceedings of the second international workshop. Edinburgh, Scotland: Nuclear Enterprises, 1983:43–8
23. Gravelle CR, Bradley DW, Cook EH, Maynard JE. Temporal patterns of ultrastructural alterations in hepatocytes of chimpanzees with experimental non-A, non-B hepatitis. J Infect Dis 1982;**145**:854–8
24. David-Ferreira JF, Manaker RA. An electron microscope study of the development of a mouse hepatitis virus in tissue culture cells. J Cell Biol 1965;**24**:57–78
25. Murphy FA. Togavirus morphology and morphogenesis. In: Schlesinger RW, ed. The togaviruses: biology, structure, replication. New York: Academic Press, 1980:241–316
26. Westaway EG. Replication of flaviviruses. In: Schlesinger RW, ed. The togaviruses: biology, structure, replication. New York: Academic Press, 1980:531–81
27. Compans RW, Choppin PW. Orthomyxoviruses and paramyxoviruses. In: Haugenau F, Dalton AJ, eds. Ultrastructure of animal viruses and bacteriophages: an atlas. New York: Academic Press, 1973:213–37
28. Amako K, Dales S. Cytopathology of mengovirus infection. II. Proliferation of membranous cisternae. Virology 1967;**32**:201–15
29. Dales S, Eggers HJ, Tamm I, Palade GE. Electron microscopic study of the formation of poliovirus. Virology 1965;**26**:379–89
30. Friedmann A, Lipton HL. Replication of Theiler's murine encephalomyelitis viruses in BHK21 cells: an electron microscopic study. Virology 1980;**101**:389–98