INFECTION AND IMMUNITY, Aug. 1983, p.816–821
0019-9567/83/080816-06$02.00/0

Vol. 41, No. 2

Inactivation of Hepatitis B Virus and Non-A, Non-B Hepatitis by Chloroform

STEPHEN M. FEINSTONE,[1]* KATHLEEN B. MIHALIK,[1] TOMOTERU KAMIMURA,[1] HARVEY J. ALTER,[2] WILLIAM T. LONDON,[3] AND ROBERT H. PURCELL[1]

Hepatitis Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205[1]; Immunology Section, Blood Bank Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20205[2]; and National Institute of Neurological and Communicative Diseases, Bethesda, Maryland 20205[3]

Received 19 January 1983/Accepted 25 April 1983

To determine whether a non-A, non-B hepatitis agent contained essential lipids, we extracted with chloroform a dilution of human plasma that contained approximately 10^4 chimpanzee infectious doses of non-A, non-B hepatitis virus and then tested for infectivity in chimpanzees. In addition, we treated a serum containing hepatitis B virus in the same way. Both of these samples were also sham extracted as controls. Known chloroform-sensitive and chloroform-resistant viruses were added directly to the hepatitis-containing serum or plasma as internal controls or to fetal calf serum as external controls and were assayed for infectivity in vitro after chloroform extraction or sham extraction. All infectivity of the diluted plasma that contained at least 10^4 chimpanzee infective doses of non-A, non-B hepatitis agent and all infectivity of the serum that contained $10^{3.5}$ chimpanzee infective doses of hepatitis B virus were destroyed by chloroform. The chloroform-sensitive control viruses were completely inactivated, but the chloroform-resistant control viruses lost less than 0.5 $\log_{10}$ of infectivity. Sham-extracted non-A, non-B hepatitis agent-containing plasma was shown to maintain its infectivity in chimpanzees that had initially been inoculated with the chloroform-extracted plasma. Thus, both hepatitis type B and non-A, non-B hepatitis appear to be caused by viruses that can be inactivated by a lipid solvent.

Non-A, non-B hepatitis (NANBH) is the major cause of transfusion-associated hepatitis in the United States. At present, less than 10% of posttransfusion cases of this disease are caused by hepatitis B virus (HBV). Of the remainder, cytomegalovirus may account for a small proportion, but the vast majority are caused by an as-yet-unidentified agent(s) (1). There is a large amount of evidence supporting the hypothosis that a transmissible agent is the cause of NANBH. This includes epidemiological studies in humans and experimental transmission studies in humans and nonhuman primates (2, 4, 6, 8, 9, 11, 13). Both chimpanzees and marmoset monkeys have been shown to be susceptible to infection by at least some NANBH agents. Although these animals are very costly and cumbersome to work with, they can be used to aid in the characterization of the infectious agent of NANBH. We have previously titrated two strains of NANBH in chimpanzees and found one, strain F (plasma sample from a patient with chronic NANBH), to have a titer of less than 10^2 chimpanzee infectious doses (CID) per ml. The second, strain H (plasma from a patient with early acute posttransfusion NANBH), had a titer of $\geq 10^6$ CID/ml (5). The availability of a high-titer NANBH inoculum enables investigators to perform certain characterization experiments that could not be done with plasma with a low titer of infectious virus. The potential interference of high concentrations of plasma lipids and proteins can be largely eliminated by dilution, and a high degree of infectivity is maintained.

One of the fundamental characteristics of viruses is whether they contain lipids as part of their structures. Viruses that do contain essential lipids can be inactivated by lipid solvents, such as ether or chloroform. Such inactivation studies have been used to help classify viruses. In this paper we describe the chloroform sensitivity of strain H of NANBH virus.

MATERIALS AND METHODS

Infectious inoculum. A human plasma designated plasma H was obtained by plasmapheresis from a patient with acute posttransfusion NANBH. Samples of undiluted plasma and 10-fold dilutions in fetal calf serum from 10^{-1} to 10^{-10} were prepared from this

plasma, divided into 1-ml quantities in vials, and stored at −70°C until they were used. This plasma has been shown to have an infectivity titer of at least 10^6 CID/ml (5); 1 ml of this plasma, undiluted ($\geqq 10^6$ CID), was thawed and treated as described below.

A human serum containing HBV strain MS-2 ($10^{7.5}$ CID/ml) also was diluted in fetal calf serum, divided, and stored in a similar manner (3). A 1-ml vial of a 10^{-2} dilution of the strain MS-2 serum ($10^{5.5}$CID) was thawed and treated as described below.

Control viruses. Representative chloroform-sensitive and -resistant viruses were selected as internal and external controls. The internal controls were added directly to 1 ml of a 1:10 dilution in sterile, phosphate-buffered saline of the NANBH plasma or to the HBV serum. The internal control viruses were selected because the human serum or plasma containing the hepatitis agents lacked antibody to the control viruses, because the control viruses did not replicate significantly in the chimpanzees which were used to assay the hepatitis viruses, and because the control viruses each could be assayed separately in the presence of the other without interference. Avian influenza virus A/pintail/Alberta/119/79 (H_4N_6) was used as the chloroform-sensitive internal control. Approximately 10^8 50% tissue culture infective doses was added to each of the hepatitis specimens. Coliphage φX174 was used as the chloroform-resistant virus, and approximately 10^{10} infectious particles were added to each of the hepatitis specimens. External control viruses were chosen because they represented typical human infectious agents and could be assayed easily. A 1-ml portion of poliovirus type I vaccine strain LSC containing approximately 10^7 50% tissue culture infective doses per ml was added to 1 ml of a 1:10 dilution of fetal calf serum, which was then diluted to a final volume of 10 ml; this preparation was used and as the chloroform-resistant external control virus. A 1-ml portion of vaccinia virus strain Elstree (10^6 50% tissue culture infective doses per ml) was added in a similar way to diluted fetal calf serum and used as the chloroform-sensitive external control virus.

Chloroform extraction. Each preparation was diluted to a final volume of 10 ml or to a 1:100 final concentration of serum or plasma. Each was then divided into two 5-ml samples in 20-ml glass, screw-capped tubes, and 0.55 ml of chloroform from a freshly opened bottle (J. T. Baker Chemical Co., Phillipsburg, N.J.) was added to one tube of each pair to make a final chloroform concentration of 10% (vol/vol). Each specimen (both those containing chloroform and those not containing chloroform) was then agitated on a Vortex mixer (Scientific Industries Inc., Bohemia, N.Y.) for 10 min at room temperature and centrifuged at 1,000 rpm for 10 min in a Sorvall 3B centrifuge with a type GSA rotor.

The aqueous phase was then carefully pipetted from the interface of each chloroform-containing sample and from any pelleted solid material in the samples not containing chloroform. These supernatants were divided into 1-ml portions and stored at −70°C until they were assayed.

Viral infectivity assays. Avian influenza virus was assayed on MDCK cells by determining cytopathic effects and by hemadsorption using guinea pig erythrocytes. Quadruplicate wells of six-well plates were inoculated for each dilution of a serial 10-fold dilution series of both the chloroform-treated and sham-treated specimens.

φX174 was assayed by decimal tube dilution in quadruplicate in L broth, using *Escherichia coli* 4704 as the host organism.

Vaccinia virus infectivity was assayed by determining cytopathic effects in Vero cells. Quadruplicate wells of six-well plates were inoculated with serial 10-fold dilutions of fetal calf serum suspensions, and the relative titers of the chloroform-treated and sham-treated specimens were compared.

Poliovirus was assayed by determining cytopathic effects in Vero cells in the manner described above for vaccinia virus.

Chimpanzee inoculations. The chimpanzees used in this study were born and raised in captivity under conditions that limited their exposure to common human and chimpanzee pathogens. They were housed in negative-pressure isolators maintained as described previously (3) and ranged in age from 49 to 69 months. Two chimpanzees (chimpanzees 889 and 947) were each inoculated intravenously with 1 ml of the chloroform-treated NANBH virus. One chimpanzee (chimpanzee 967) was inoculated with 1 ml of chloroform-treated HBV. The chimpanzees were monitored for hepatitis by determining alanine aminotransferase (ALT) levels and aspartate aminotransferase levels weekly. Hepatitis B surface antigen, antibody to hepatitis B surface antigen, and antibody to hepatitis B core antigen were also measured in the plasma from strain MS-2-inoculated chimpanzee by using commercial radioimmunoassays (Ausria, Ausab, and Corab; Abbott Laboratories, North Chicago, Ill.). In addition, percutaneous liver biopsies were obtained weekly from all chimpanzees. These biopsies were divided into three pieces and were fixed in 10% buffered Formalin for routine histological examination, fixed in glutaraldehyde for electron microscopy, and snap-frozen for immunofluorescence.

Diagnosis of hepatitis. Hepatitis was diagnosed in a chimpanzee if the ALT level rose to more than twice the upper limit of normal (considered to be 40 IU/liter). Selected liver biopsies were examined by electron microscopy, using methods described previously (12).

RESULTS

All of the internal and external control viruses reacted to the chloroform treatment as predicted (Table 1). The avian influenza virus and vaccinia virus were totally inactivated by chloroform, whereas poliovirus and φX174 were unaffected.

Figure 1 shows the weekly ALT levels in the two chimpanzees inoculated with chloroform-treated NANBH virus (chimpanzees 889 and 947) and the one chimpanzee inoculated with chloroform-treated HBV (chimpanzee 967). None of these animals developed biochemical evidence of hepatitis. Chimpanzee 967, which was inoculated with HBV, did develop a weak antibody to hepatitis B surface antigen (maximum S/N, 4.2) but not an antibody to hepatitis B core antigen. This is typical of a response to hepatitis B vaccine and indicates that hepatitis B surface antigen in the inoculum and not infec-

TABLE 1. Chloroform extraction of control viruses

Prepn[a]	Virus titer ($\log_{10}$ 50% infectious doses per 0.1 ml)	
	$CHCl_3$ extraction	Sham extraction
Internal controls		
φX174 in plasma H	9.0	9.5
φX174 in strain MS-2 serum or fetal calf serum	9.0	9.5
Avian influenza virus in plasma H	≦0.5	5.25
Avian influenza virus in strain MS-2 serum or fetal calf serum	≦0.5	5.5
External controls		
Polio virus type 1 in fetal calf serum	6.5	6.5
Vaccinia virus in fetal calf serum	≦0.5	5.5

[a] The final serum or plasma concentration was 1:100 for each virus suspension.

tious virus was responsible for the induction of the antibody to hepatitis B surface antigen. At that time, chimpanzee 967 was probably resistant to infection with live HBV due to its acquisition of antibody to hepatitis B surface antigen. Therefore, challenge with sham-treated HBV was postponed (see below). Instead, 6 months after the initial inoculations, all three of the animals were inoculated with sham-treated NANBH. As Fig. 1 shows, chimpanzee 967 (which had no prior exposure to NANBH virus) developed NANBH with a 5-week incubation period. Chimpanzee 889 (which was initially inoculated with chloroform-treated NANBH virus) also developed NANBH with an incubation period of about 5 weeks after rechallenge with the sham-treated NANBH virus. Chimpanzee 947 did not develop elevated ALT levels diagnostic of hepatitis after inoculation with either chloroform-treated or sham-treated NANBH virus.

Liver biopsies were obtained at weekly inter-

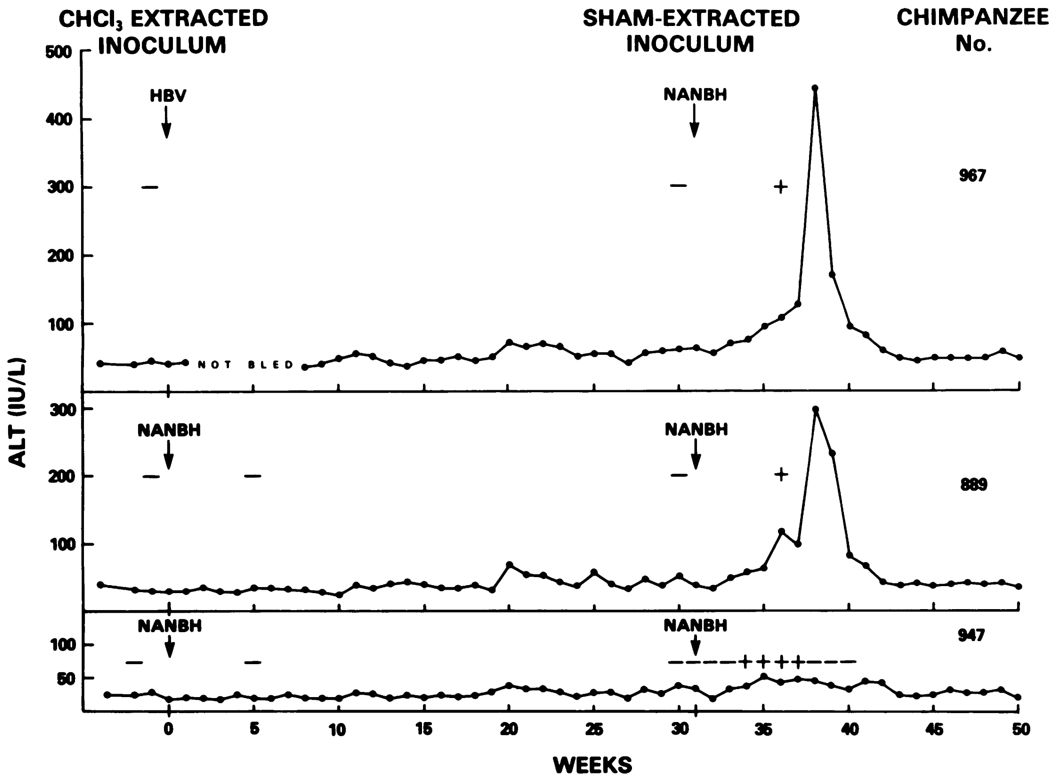

FIG. 1. Weekly ALT levels in chimpanzees inoculated initially which chloroform-extracted HBV strain MS-2 (chimpanzee 967) or NANBH strain H (chimpanzees 889 and 947). At 31 weeks after the initial inoculation, all of the animals were inoculated with sham-extracted NANBH plasma. Chimpanzees 967 and 889 developed elevated ALT levels diagnostic of hepatitis, but chimpanzee 947 did not. Electron microscopy of selected liver biopsies revealed the presence of cytoplasmic tubular structures that are typical of NANBH in all three animals, including chimpanzee 947, after inoculation with the sham-extracted NANBH plasma, indicating that all three were probably infected with NANBH virus. The plus signs indicate biopsies that were examined that contained these structures, and the minus signs indicate biopsies that did not contain the structures.

vals from the three chimpanzees throughout the study. Selected biopsies from each animal were coded and then examined by electron microscopy for the presence of the distinctive cytoplasmic tubular structures that have been associated with NANBH in chimpanzees (5, 12). The biopsies examined were obtained before inoculation, 5 weeks after primary inoculation with chloroform-extracted NANBH virus or HBV, just before challenge with sham-treated NANBH plasma, and 5 weeks after inoculation with sham-extracted plasma. In addition, all biopsies from chimpanzee 947 for the first 10 weeks beginning at the time of challenge with sham-treated NANBH virus were examined to determine whether there was ultrastructural evidence for inapparent infection with NANBH virus in this chimpanzee, which did not develop elevated ALT levels diagnostic of hepatitis. All biopsies taken from all of the animals studied in the period before challenge with sham-inactivated NANBH plasma were normal. Chimpanzees 889 and 967 developed the characteristic cytoplasmic tubular structures in biopsies taken 5 weeks after challenge with the sham-treated NANBH plasma, at a time when both of these animals had elevated ALT levels. Biopsies from chimpanzee 947 that did not develop elevated ALT levels were found to contain the cytoplasmic structures from week 3 through week 6 after challenge with sham-extracted NANBH plasma (Fig. 1 and 2). Therefore, even chimpanzee 947 had ultrastructural evidence of infection after inoculation with the sham-extracted NANBH virus.

At 82 weeks after initial inoculation with $CHCl_3$-tested HBV, chimpanzee 967 was reevaluated for antibody to hepatitis B surface antigen and antibody to hepatitis B core antigen and found to be negative. This animal was then intravenously inoculated with $10^{3.5}$ CID of untreated HBV (data not shown). Chimpanzee 967 developed typical markers of HBV infection beginning 6 weeks after inoculation, thus con-

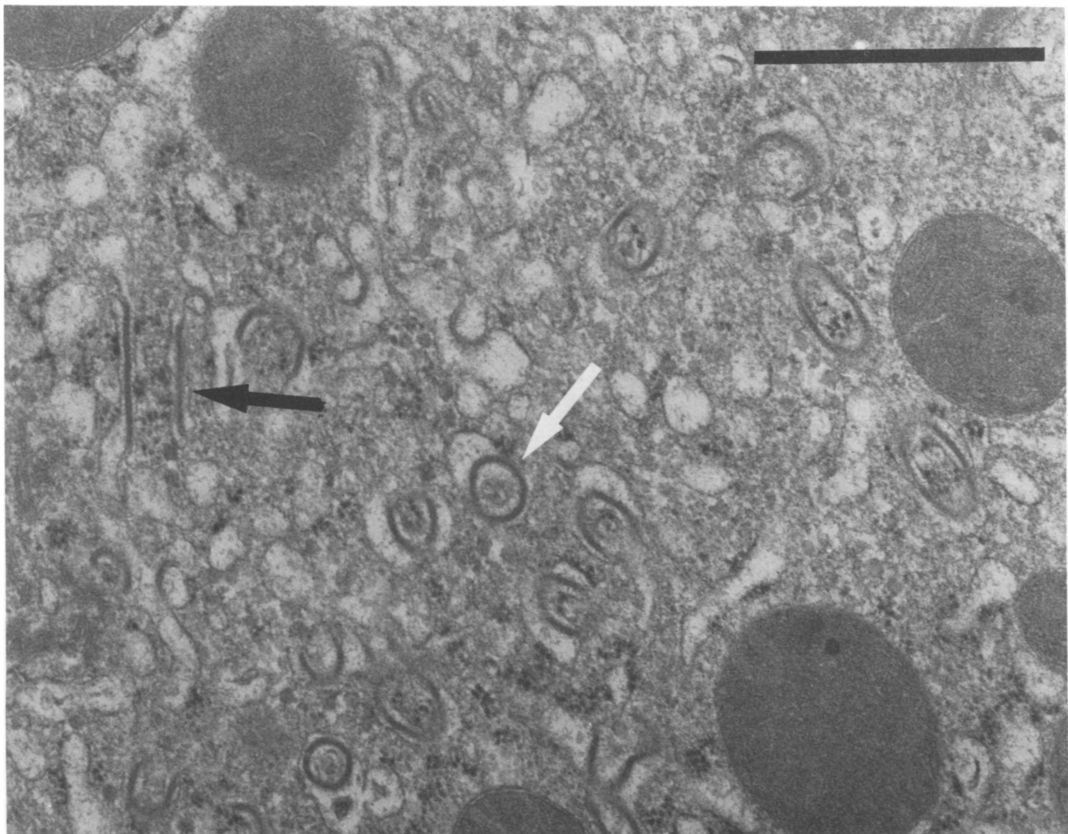

FIG. 2. Electron micrograph of a liver biopsy from chimpanzee 947 5 weeks after inoculation with sham-extracted NANBH plasma. The hepatocyte cytoplasm contains the tubular structures typical of NANBH in chimpanzees. The white arrow indicates a tubule cut in cross-section, and the black arrow indicates a longitudinally cut tubule. Bar = 1 μm.

firming that $CHCl_3$ did inactivate HBV in the initial experiment in this chimpanzee.

DISCUSSION

Sensitivity to organic solvents has been an important criterion for classifying viruses. Diethyl ether has been used to determine whether a virus contains essential lipids. However, ether is a relatively poor lipid solvent, and poxviruses have been found to be partially resistant to ether extraction. On the other hand, chloroform is a more potent lipid solvent, and it more efficiently inactivates poxviruses (10). Thus, viruses that contain essential lipids are inactivated by chloroform, whereas viruses without lipids generally are resistant. However, some viruses without lipids, such as reovirus, may be partially inactivated by chloroform extraction. This inactivation may be due to the denaturation of some proteins by chloroform or to surface denaturation at the chloroform-water interface (10). Therefore, even though sensitivity to chloroform does not prove that a virus contains essential lipids, it strongly suggests that it does. Conversely, if a virus is resistant to chloroform extraction, it is highly unlikely that it contains lipids.

The agent(s) responsible for NANBH is poorly characterized due to the lack of an in vitro cultivation system or a confirmed serologial assay. Characterization has depended on utilization of the chimpanzee or marmoset animal model. The relatively high-titer plasma H makes certain characterization experiments possible that cannot be performed with a low-titer inoculum. In this study we diluted the starting plasma to a final concentration of 10^{-2} to remove most of the effect of the high concentrations of plasma proteins and lipids on the chloroform extraction. This allowed us to determine whether at least 10^4 CID of virus could be inactivated by chloroform. Other chimpanzee inocula that have been described have relatively low titers (usually 10^3 CID/ml or below), and these inocula are less useful for many characterization experiments (14).

HBV does not contain a bilayer lipoprotein membrane envelope, but the virus coat does contain lipoprotein (7). Therefore, it was assumed that HBV would be sensitive to chloroform, but infectivity experiments with chloroform-treated HBV have not been described previously. We have shown that $10^{3.5}$ CID/ml of HBV are completely inactivated by treatment with chloroform. Since 10^4 CID of NANBH strain H were also inactivated by chloroform, we concluded that this agent also contains essential lipids.

If both HBV and NANBH virus can be inactivated by chloroform, it should be possible to use treatment with chloroform or other lipid solvents to eliminate these agents from certain blood products that can withstand such treatment. Factor VIII and other clotting factor concentrates that are prepared from large pools of plasma are very frequently contaminated by both HBV and NANBH virus, although HBV contamination has become less important since careful screening of donor plasma before pooling was initiated. It may be possible to eliminate any residual infectious HBV from these pools by chloroform treatment. In addition, at least the form of NANBH virus that we studied in this work would also be inactivated by the chloroform. Thus, the major health hazard of these transfusion products potentially could be eliminated.

Further characterization studies on NANBH virus in animal models may help reveal the nature of the agent and establish reliable methods of detection.

LITERATURE CITED

1. **Alter, H. J., R. H. Purcell, S. M. Feinstone, and G. E. Tegtmeier.** 1982. Non-A, non-B hepatitis: its relationship to cytomegalovirus, to chronic hepatitis, and to direct and indirect test methods, p. 279–294. *In* W. Szmuness, H. J. Alter, and J. E. Maynard (ed.), Viral Hepatitis International Symposium. The Franklin Institute Press, Philadelphia.
2. **Alter, H. J., R. H. Purcell, P. V. Holland, and H. Popper.** 1978. Transmissible agent in non-A, non-B hepatitis. Lancet i:459–463.
3. **Barker, L. F., J. E. Maynard, R. H. Purcell, J. H. Hoofnagle, K. R. Berquist, W. T. London, R. J. Gerety, and D. H. Krushak.** 1975. Hepatitis B virus infection in chimpanzees: titration of subtypes. J. Infect. Dis. **132:**451–458.
4. **Dienstag, J. L., W. A. Krotoski, W. A. Howard, R. H. Purcell, F. A. Neva, J. T. Galambos, and R. H. Glew.** 1981. Non-A, non-B hepatitis after experimental transmission of malaria by inoculation of blood. J. Infect. Dis. **143:**200–208.
5. **Feinstone, S. M., H. J. Alter, H. P. Dienes, Y. Shimizu, H. Popper, D. Blackmore, D. Sly, W. T. London, and R. H. Purcell.** 1981. Non-A, non-B hepatitis in chimpanzees and marmosets. J. Infect. Dis. **144:**588–598.
6. **Feinstone, S. M., A. Z. Kapikian, R. H. Purcell, H. J. Alter, and P. V. Holland.** 1975. Transfusion-associated hepatitis not due to viral hepatitis type A or B. N. Engl. J. Med. **292:**767–770.
7. **Gerin, J. L., R. H. Purcell, M. D. Hoggan, P. V. Holland, and R. M. Chanock.** 1969. Biophysical properties of Australia antigen. J. Virol. **4:**763–768.
8. **Hollinger, F. B., G. L. Gitnick, R. D. Aach, W. Szmuness, J. W. Mosley, C. E. Stevens, R. L. Peters, J. M. Weiner, J. B. Werch, and J. J. Lander.** 1978. Non-A, non-B hepatitis transmission in chimpanzees: a project of the transfusion-transmitted viruses study group. Intervirology **10:**60–68.
9. **Hoofnagle, J. H., R. J. Gerety, E. Tabor, S. M. Feinstone, L. F. Barker, and R. H. Purcell.** 1977. Transmission of non-A, non-B hepatitis. Ann. Intern. Med. **87:**14–20.
10. **Philipson, L.** 1967. Water-organic solvent phase systems, p. 235–244. *In* K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. 2. Academic Press, Inc., New York.
11. **Prince, A. M., B. Brotman, G. F. Grady, W. J. Kuhns, C. Hazzi, R. W. Levine, and S. J. Millian.** 1974. Long-incubation post-transfusion hepatitis without serological evi-

dence of exposure to hepatitis-B virus. Lancet **ii**:241–246.
12. **Shimizu, Y. K., S. M. Feinstone, R. H. Purcell, H. J. Alter, and W. T. London.** 1979. Non-A, non-B hepatitis: ultrastructural evidence for two agents in experimentally infected chimpanzees. Science **205**:197–200.
13. **Tabor, E., and R. J. Gerety.** 1980. Inactivation of an agent of human non-A, non-B hepatitis by formalin. J. Infect. Dis. **142**:767–770.
14. **Yoshizawa, H., Y. Itoh, S. Iwakiri, K. Kitajima, A. Tanaka, T. Tachibana, T. Nakamura, Y. Miyakawa, and M. Mayumi.** 1982. Non-A, non-B (type 1) hepatitis agent capable of inducing tubular ultrastructures in the hepatocyte cytoplasm of chimpanzees: inactivation by formalin and heat. Gastroenterology **82**:502–506.