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Production of antibody associated with non-A, non-B hepatitis in a chimpanzee lymphoblastoid cell line established by *in vitro* transformation with Epstein–Barr virus

(immunofluorescence/chimpanzee livers/microtubular aggregates)

YOHKO K. SHIMIZU*, MASASHI OOMURA*, KENGI ABE*, MASATUNE UNO†, EI YAMADA†, YASUSHI ONO†, AND TOSHIO SHIKATA*

Departments of *Pathology and †Microbiology, Nihon University School of Medicine, Itabashiku, Tokyo 173, Japan

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ABSTRACT **A continuous cell line of chimpanzee lymphocytes producing an antibody specifically associated with non-A, non-B hepatitis (NANB) was established. Peripheral blood lymphocytes of a chimpanzee convalescent from experimental infection with NANB hepatitis were transformed *in vitro* by Epstein–Barr virus infection into lymphoblastoid cell lines. Supernatants of the cell cultures were screened by immunofluorescence for antibody activity against the liver tissue of a chimpanzee with NANB hepatitis. Nineteen of the 1402 cultures were found to be positive for the activity. Ten of these 19 gave cytoplasmic reactions and the remaining 9 gave nuclear reactions in hepatocytes. One culture (48-1) stably producing the antibody was further characterized. The antibody produced in 48-1 was IgM and gave granular cytoplasmic reactions in hepatocytes. Cloning of 48-1 was performed by the soft agar method and cloned cell lines stably producing the antibody were obtained. The 48-1 antibody reacted with liver biopsy specimens from 12 chimpanzees obtained during the acute or chronic phase of hepatitis caused by five different NANB strains, but not with biopsy specimens from chimpanzees with hepatitis A or B or from normal chimpanzees. In addition, examinations of serial liver biopsy specimens obtained from 2 chimpanzees experimentally infected with NANB hepatitis demonstrated that the antibody reacted with the biopsies obtained during the preacute, acute, and chronic hepatitis, but not with those obtained before inoculation, early incubation period, or during convalescence. The present results indicate the specific association of the antibody with NANB hepatitis. Immunoelectron microscopy revealed that the antibody reacted with the microtubular aggregates identical to those previously described in a patient and chimpanzees with NANB hepatitis.**

Presently, up to 90% of the cases of posttransfusion hepatitis are not related to hepatitis A or B viruses or to other known viruses that cause liver disease. This newly recognized form of hepatitis has been designated as "non-A, non-B hepatitis" (NANB hepatitis), since more than one agent may be involved. The transmissible nature of the disease has been demonstrated by inoculating serum or plasma from patients with NANB hepatitis into chimpanzees (1–3). Electron microscopic studies of livers from infected chimpanzees revealed characteristic ultrastructural alterations in hepatocytes (4, 5). However, to date a NANB hepatitis virus has not been identified nor has a specific serological assay that is applicable to diagnosis and blood donor screening been established.

In an attempt to detect antibodies associated with NANB hepatitis, we employed the Epstein–Barr virus (EBV) transformation method introduced by one of us (6) and Steinitz *et al.* (7).

This method is based on the fact that B lymphocytes can be transformed *in vitro* by EBV infection into lymphoblastoid cell lines capable of producing specific antibodies. The successful production, using this method, of antibodies against a variety of antigens, such as Rh antigen D (8), diphtheria toxoid (9), and hepatitis B surface antigen,‡ has been reported.

We describe in this paper the establishment by the EBV transformation method of a continuous cell line of chimpanzee lymphocytes producing an antibody specifically associated with NANB hepatitis.

MATERIALS AND METHODS

Preparation of Lymphocytes. Lymphocytes were isolated by the Ficoll-Isopaque method from peripheral blood of chimpanzee 48 who was in the convalescent phase of experimental infection with the strain F of NANB hepatitis (kindly provided by R. H. Purcell, National Institutes of Health, Bethesda, MD).

Establishment of Lymphoblastoid Cell Lines. Lymphocytes (2×10^7) were incubated at 37°C for 60 min in 20 ml of supernatant of the EBV (B95-8 strain) producing cell line (10). The multiplicity of infection was calculated to be ≈0.1. The cells were then distributed at cell concentrations of 1×10^4, 2.5×10^4, and 5×10^4 cells per 0.2 ml per well in 96-well microculture plates in medium RPMI 1640 containing 20% fetal calf serum. Half of the volume of media was changed every 4 days as described (11).

Screening for Antibody Activity. The culture supernatants of EBV transformants were individually tested for antibody activity by immunofluorescence (IF) using sections of liver tissue of chimpanzee 34. Chimpanzee 34 had been inoculated with the acute phase plasma from chimpanzee 61 (the fourth passage of the strain F of NANB hepatitis in chimpanzees) and had developed histological evidence of hepatitis 5 wk after inoculation, although serum alanine aminotransferase activity remained in the normal range. Chimpanzee 34 died accidentally 8 wk after inoculation and the necropsy liver specimen was used for the screening of lymphocytes.

IF Tests. Sections (4 μm) of liver tissue (unfixed) were incubated with undiluted culture supernatants for 6 hr at room temperature, washed with phosphate-buffered saline, and incubated with a 1:100 dilution of fluoresceinated anti-human Ig or IgM (Medical and Biological Laboratories, Nagoya, Japan) at 4°C overnight.

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Abbreviations: EBV, Epstein–Barr virus; NANB, non-A, non-B; IF, immunofluorescence; EM, electron microscopy.

‡Ono, Y., Nakagomi, A., Nakae, S., Yoshie, O., Shiroishi, H. & Ishida, N., Fourth International Congress of Immunology, July 21–26, 1980, Paris, abstr. 9.6.08.

Cloning of the 48-1 Culture. The soft agar method was employed (12). One-tenth milliliter of cell suspension (1×10^4 or 5×10^3 cells per 6-cm dish) was mixed with 3 ml of 0.3% melted agar in medium RPMI 1640 supplemented with 20% fetal calf serum. Cells were then placed on a layer of 4 ml of 0.5% agar in a Petri dish. The dish was incubated at 37°C in a CO_2 incubator. After 2 wk of culture, developed colonies were picked up by Pasteur pipettes and transferred to the culture medium in 96-well microculture plates. The culture supernatants of cloned cell cultures were assayed by IF for production of the antibody.

Liver Tissues Examined. The liver tissues tested by IF consisted of liver biopsy specimens obtained from 13 chimpanzees inoculated with the NANB hepatitis materials (details of these 13 chimpanzees are shown in Table 1), 4 chimpanzees acutely or persistently infected with hepatitis B virus, 1 chimpanzee with fulminant hepatitis A, and 10 uninfected chimpanzees. In addition, the 48-1 antibody (described in *Results*) was tested by IF on serial liver biopsy specimens obtained from 2 chimpanzees (38 and 61) who were inoculated with the strain F of NANB hepatitis (Table 1). Chimpanzee 61 developed chronic liver disease following acute hepatitis and a pool of plasma (48–51 wk) was found to cause hepatitis in a recipient chimpanzee, thus documenting persistent infection of the NANB hepatitis agent.

Standard Thin-Section Electron Microscopy (EM). A piece of liver tissue was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3), postfixed in 1% osmium tetroxide in the same buffer, dehydrated in ethanol, infiltrated with propylene oxide, and embedded in Epon 812 mixture. Ultrathin sections were stained with uranyl acetate and lead citrate.

Immunoperoxidase EM. Sections of the liver tissue were fixed in 4% paraformaldehyde solution at 4°C for 20 min, incubated with undiluted supernatant of the 48-1 culture at room temperature for 6 hr, washed, and incubated with a 1:100 dilution of peroxidase-conjugated Fab' anti-human IgM (Medical and Biological Laboratories) at 4°C overnight. The tissue sections were then fixed in 2.5% glutaraldehyde solution at 4°C for 20 min, incubated in 0.02% solution of 3,3'-diaminobenzidine containing 0.002% of hydrogen peroxide for 10 min, dehydrated in graded ethanol to 100%, and embedded in Epon 812 mixture. The thin sections of Epon blocks were examined under the light microscope and positively stained areas were selected. Ultrathin sections were observed by EM without additional staining.

RESULTS

Peripheral lymphocytes from chimpanzee 48, convalescent from infection with the strain F of NANB hepatitis agent, were inoculated with EBV and cultured in 96-well microculture plates. Continuous cell cultures emerged within 4 wk after the initiation of culture with an average frequency of 96% in the three experiments (Table 2). At wk 4, the culture supernatants were individually harvested and tested by IF for antibodies against sections of liver tissue obtained from chimpanzee 34, inoculated with the strain F of NANB hepatitis.

As shown in Table 2, antibody activity was detected in 19 of the 1402 supernatants. Ten of the 19 positive supernatants gave cytoplasmic granular staining and the remaining 9 produced nuclear staining in hepatocytes. The positive cultures were then passaged into successively larger volumes and the activity in the supernatants was examined at weekly intervals. The activity in 17 of the 19 positive cultures gradually decreased and became undetectable within 10 wk. However, 2 cultures that gave cytoplasmic reactions continuously produced IF antibodies. One of these 2, 48-1, was chosen to be further characterized. Fig. 1 shows positive IF in hepatocytes of chimpanzee 34 by the 48-1 antibody. The granular staining was located in the cytoplasm and seen in almost every hepatocyte. The titer of the antibody activity in 48-1 measured by IF was 1:64. It was determined by IF and immunodiffusion tests that the immunoglobulin class of the antibody was IgM.

Cloning (soft agar method) was carried out on the 48-1 culture. Cells were seeded in 0.3% agar without feeder cells and cultured for 2 wk. Seventy-two colonies were isolated, but only 10 grew into cell lines. Of the 10 cloned cell lines, 8 were positive for IF antibody activity. To investigate the specificity of the antibody for NANB hepatitis, the supernatant of 48-1 was tested by IF on liver biopsy specimens from chimpanzees with hepatitis A, B, or NANB or from normal chimpanzees. Liver biopsy specimens from 13 chimpanzees with NANB hepatitis were examined. The antibody reacted

Table 1. Description of chimpanzees with experimental NANB hepatitis studied by IF

Chimpanzee no.	Source of inoculum	ALT elevation, wk	Maximal ALT, Karmen units	Liver histology	Tubular structures*	Cytoplasmic IF by the 48-1 antibody
48	Chimpanzee 884 (wk 8)†	6, 9–14	74	+	+	NT
11	Chimpanzee 884 (wk 8)	2–5	45	+	+	+
61	Chimpanzee 48 (wk 5–12)	6, 11–15	269	+	+	+
38	Chimpanzee 48 (wk 5–12)	10–15	149	+	+	+
34‡	Chimpanzee 61 (wk 5–12)			+	+	+
63	Chimpanzee 61 (wk 5–12)	7–13	172	+	NT	+
41	Chimpanzee 61 (wk 48–51)	20–23	52	+	+	+
42	Patient H (acute phase)§	20	42	+	+	+
54	Patient Y (acute phase)¶	13	39	+	+	+
59	Patient F (acute phase)¶	No elevation		+	+	+
40	Chimpanzee 59 (wk 93, 102, 112)	No elevation		+	+	+
82	Patient S (acute phase)§	18–19	230	+	+	+
84	Chimpanzee 82 (wk 14)	No elevation		+	+	+
12	Patient B (acute phase)§	5–8	45	+	−	−

ALT, alanine aminotransferase; NT, not tested.
*Ultrastructures described previously for chimpanzees infected with the strain F of NANB hepatitis.
†The second passage of the strain F of NANB hepatitis in chimpanzees (provided by R. H. Purcell).
‡Accidental death at 8 wk after the inoculation.
¶Derived from an outbreak of NANB hepatitis in Shimizu City, Japan.
§Patient with posttransfusion NANB hepatitis.

Table 2. Frequency of transformants and positive cultures

Experiment	Inoculated cells, no. per well	Frequency of transformants (%)	Frequency of positive cultures (%)	
			Cytoplasm	Nucleus
1	5.0×10^4	245/245 (100)	2/245 (0.8)	3/245 (1.2)
	2.5×10^4	205/290 (71)	0/205 (0)	1/205 (0.4)
2	5.0×10^4	218/218 (100)	2/218 (0.9)	2/218 (0.9)
	2.5×10^4	300/300 (100)	2/300 (0.7)	2/300 (0.7)
3	5.0×10^4	117/117 (100)	3/117 (2.5)	1/117 (0)
	2.5×10^4	117/117 (100)	0/117 (0)	1/117 (0)
	1.0×10^4	960/960 (100)	1/200 (0.5)	1/200 (0.5)
Total		2162/2247 (96)	10/1402 (0.7)	9/1402 (0.6)

After 4 wk of culture, the supernatants were tested by IF on the liver of chimpanzee 34.

with biopsies obtained during acute or chronic hepatitis (31 samples) from 6 chimpanzees inoculated with the strain F of NANB hepatitis and with 12 samples from 4 chimpanzees, each inoculated with a specimen from a separate patient with NANB hepatitis, and 2 chimpanzees inoculated with serum from one of the latter chimpanzees. Thus, liver biopsy specimens that reacted with the 48-1 antibody were derived from chimpanzees inoculated with five distinct strains of NANB. The liver of a chimpanzee inoculated with a specimen from a fifth patient with NANB hepatitis was negative for the IF reaction (Table 1). The antibody did not react with liver biopsy specimens from 4 chimpanzees acutely or chronically infected with hepatitis B virus, 1 chimpanzee with fulminant hepatitis A, or 10 uninfected chimpanzees. In addition, the antibody was tested on serial liver biopsy specimens from chimpanzees 38 and 61. As shown in Fig. 2, the antibody detected cytoplasmic antigens in biopsies obtained 4–13 wk after inoculation of chimpanzee 38; a liver biopsy taken prior to inoculation and biopsies before wk 4 or after wk 13 were negative. In chimpanzee 61, who developed chronic persistent hepatitis with viremia, the cytoplasmic antigens became detectable at wk 4 and remained in hepatocytes as long as 153 wk.

To determine the ultrastructure of the cytoplasmic antigen reacting with this antibody, standard thin-section EM and immunoperoxidase EM were carried out on the liver tissue of chimpanzee 34. By standard thin-section EM, the presence of tubular structures and microtubular aggregates was detected in the cytoplasm of hepatocytes. They were morphologically identical to those described previously for chimpanzees with experimental NANB hepatitis. Microtubular aggregates were often found in the vicinity of the nucleus and surrounded by tubular structures. Fig. 3 shows an aggregate of microtubules.

When viewed under the light microscope, immunoperoxidase staining appeared to be granular and located in the cytoplasm of hepatocytes. Such positively stained areas were selected for examination by EM. Aggregates of reac-

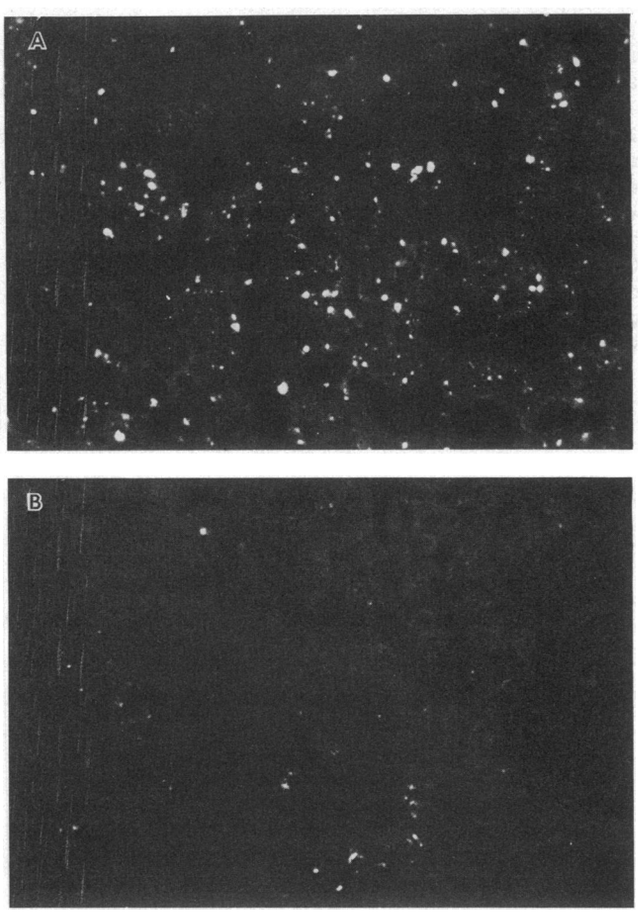

FIG. 1. Liver tissue from chimpanzee 34 obtained 8 wk after inoculation with the strain F of NANB hepatitis. (*A*) Positive cytoplasmic fluorescence with the 48-1 antibody; (*B*) abolition of fluorescence after absorption of the 48-1 antibody with the IF-positive liver extract. (×300.)

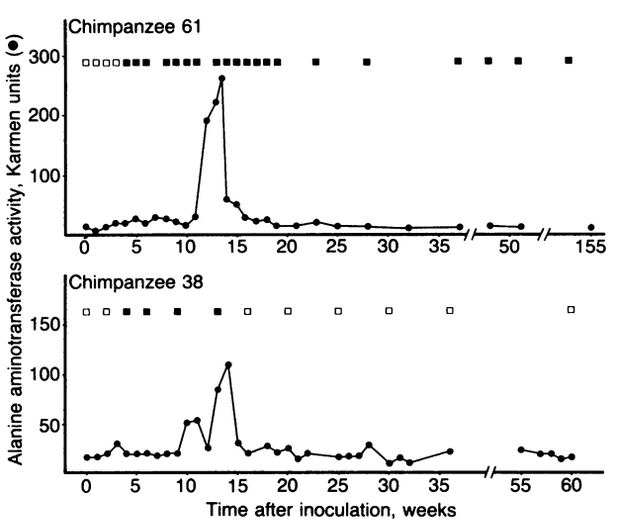

FIG. 2. Alanine aminotransferase activity in serum (●) and cytoplasmic fluorescence present (■) or absent (□) in hepatocytes following inoculation with the strain F of NANB hepatitis in 2 chimpanzees (61 and 38). Normal alanine aminotransferase activity = 30 Karmen units.

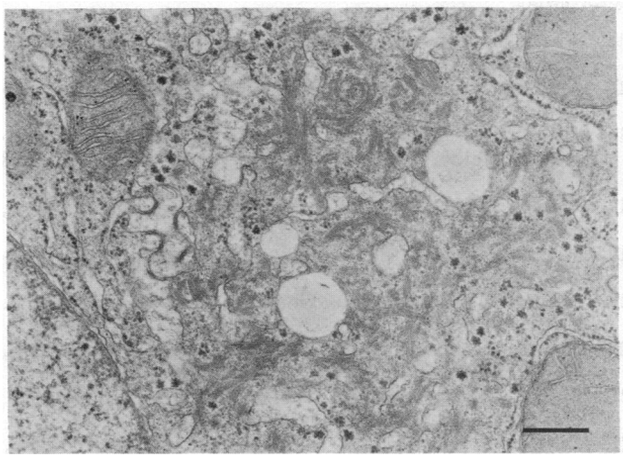

FIG. 3. An aggregate of microtubules in the cytoplasm of a hepatocyte from chimpanzee 34. (Standard thin-section EM; bar = 500 nm.)

tion products, indicating the binding of the antibody, were observed in the cytoplasm of hepatocytes. Higher magnification of a reaction product demonstrated that the antibody reacted with the microtubules (Fig. 4). Cytoplasmic tubular structures were negative for immunoperoxidase staining.

DISCUSSION

Despite intensive research by many laboratories, there is as yet no antigen–antibody system generally accepted as being specific for NANB hepatitis. Approaches that were useful in the studies of hepatitis A and B have been applied to the search for antigens and antibodies related to NANB hepatitis. The methods include immunodiffusion (13–15), RIA (16), ELISA (17), counterelectrophoresis (18), and IF (19, 20). The results of these tests, however, have been controversial. Based on our data and data of others that chimpanzees infected with NANB hepatitis frequently developed persistent liver disease with viremia, it is likely that serum or plasma from the convalescent phase of NANB hepatitis may not contain sufficient antibodies for these serological assays.

As a new approach to the detection of specific antibodies of NANB hepatitis, we applied the EBV transformation method, which is based on the fact that EBV transforms B lymphocytes of humans and marmoset monkeys *in vitro* into lymphoblastoid cell lines that synthesize and secrete immunoglobulins. In addition to humans and marmosets, our preliminary experiments showed that chimpanzee lymphocytes were also transformed by EBV infection into lymphoblastoid cell lines capable of producing antibodies. Therefore, we started our experiments with chimpanzee lymphocytes. We report in this paper the successful production of an antibody specifically associated with NANB hepatitis in a chimpanzee lymphoblastoid cell line established by *in vitro* transformation with EBV. The antibody reacted with liver biopsy specimens from chimpanzees with NANB hepatitis, but not with such specimens from chimpanzees with hepatitis A or B or from normal chimpanzees. In addition, examination of serial liver biopsies from chimpanzees infected with NANB hepatitis revealed that the antibody reacted with biopsies obtained during the preacute, acute, and chronic phases of hepatitis, but not with biopsies taken before inoculation or during convalescence. Thus, the results indicated the close association of the antibody with NANB hepatitis.

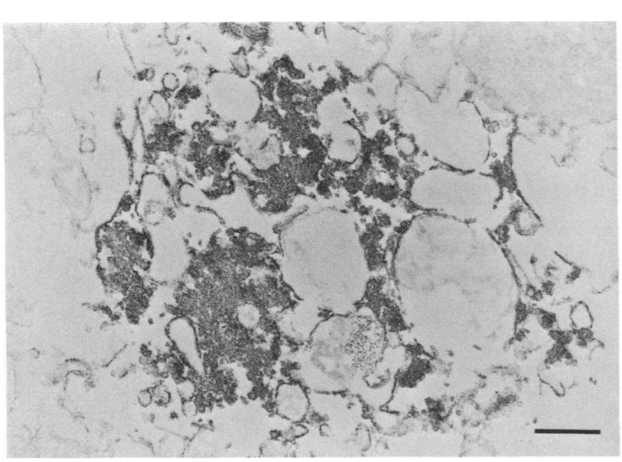

FIG. 4. Ultrastructural localization of the antigen reacting with the 48-1 antibody in a hepatocyte from chimpanzee 34. Reaction products, indicating the binding of the antibody, are seen on the microtubules. (Immunoperoxidase EM; bar = 500 nm.)

The results of the preliminary tests of the antibody on liver biopsies from human patients with hepatitis further indicated the specific association of the antibody with NANB hepatitis. The antibody reacted with liver biopsies from human patients with acute (8/13) and chronic (1/18) NANB hepatitis, but not with biopsies from patients with hepatitis A (0/6) or hepatitis B (0/27). However, it remains unknown whether the antibody is directed against the infectious agent or a host antigen induced by the agent.

Several ultrastructural alterations have been described for NANB hepatitis in patients and chimpanzees. We reported previously two different types of ultrastructural hepatic changes in chimpanzees with experimental NANB hepatitis—namely, cytoplasmic tubular structures and aggregates of nuclear particles. Subsequently, Pfeifer *et al.* (5) described two additional cytoplasmic changes in infected chimpanzee livers—microtubular aggregates and sponge-like inclusions. Nuclear particles and microtubular aggregates (21) have also been recognized in hepatocytes from human patients with NANB hepatitis but the specificity of the former has been questioned (22).

By immunoperoxidase EM, the antibody was demonstrated to react with microtubular aggregates in chimpanzee hepatocytes. The relationship of the microtubules to the infectious agent of NANB hepatitis, however, remains to be determined.

Recently, the finding of microtubules similar to those described herein was reported in a chimpanzee with δ-agent-associated hepatitis (23). The antigenic relationship between the microtubules of NANB hepatitis and those of δ-hepatitis remains to be examined by EM.

We have successfully cultured 48-1 in large quantities and purified sufficient antibodies for the development of RIA or ELISA (unpublished data). We have also tried this EBV method on lymphocytes from a patient convalescent from NANB hepatitis whose acute serum produced hepatitis in a recipient chimpanzee who developed ultrastructural changes identical to those of the strain F of NANB hepatitis and obtained a lymphoblastoid cell line producing an antibody with similar specificity. These results will be reported separately.

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