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Establishment of a human T-cell clone cytotoxic for both autologous and allogeneic hepatocytes from chronic hepatitis patients with type non-A, non-B virus

(hepatitis diagnosis/vaccine development)

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ABSTRACT **A human T-cell clone (TA-NB-2) that could lyse both autologous and allogeneic hepatocytes from chronic hepatitis patients with type non-A, non-B virus (NANB) was established. This clone produced $CD3^+$ $CD8^+$ cytotoxic T lymphocytes and expressed an antigen specific for α and β subunits of T-cell receptor. The cytotoxic activity of the clone was abrogated by incubation with anti-CD3 monoclonal antibody. Anti-HLA monoclonal antibodies did not block the lysis of the target hepatocytes by TA-NB-2 cells. The cytotoxicity of TA-NB-2 clone against hepatocytes from patients with chronic NANB hepatitis was 39.8 ± 13.2% (mean ± SD; n = 17) (range, 14.2–60.5%), whereas that against hepatocytes from control patients with chronic type-B hepatitis, acute hepatitis B, acute hepatitis A, or alcoholic liver cirrhosis was 4.0 ± 7.7% (n = 12) (range, −10.8 to 14.0%). The results suggest that TA-NB-2 cells specifically recognize a hepatitis NANB-related antigen expressed on hepatitis NANB-infected hepatocytes by T-cell receptor and that the recognition is not restricted by the major histocompatibility complex antigens. The results also suggest that most, if not all, cases of chronic hepatitis due to NANB are caused by one agent; TA-NB-2 clone may be useful as a tool to identify this particular hepatitis-related antigen.**

Although it is difficult to assess the frequency of chronic hepatitis of type non-A, non-B virus (NANB) without a specific serological test, NANB-associated hepatitis (NANB hepatitis) has been considered to cause a proportion of chronic hepatitis and cirrhosis (1). NANB hepatitis accounts for 85–95% of posttransfusion hepatitis, and the incidence of NANB hepatitis in recipients of blood from volunteer donors has been reported to be 7–10%. NANB hepatitis is also transmitted by blood products, including factor VIII concentrates, factor IX concentrates, fibrinogen preparation, and immunoglobulin preparation. Patients with posttransfusion NANB hepatitis frequently develop chronic hepatitis, which, in turn, progresses to cirrhosis in ≈20% of the cases. NANB hepatitis also occurs sporadically without known exposure to blood or blood products, and the hepatitis may be caused by the same agent as that responsible for posttransfusion NANB hepatitis. In addition, the existence of asymptomatic NANB hepatitis virus carriers has been also suggested.

Several antigen–antibody systems for diagnosis of NANB hepatitis have been reported, but none of them has been universally accepted (2). Thus the diagnosis of NANB hepatitis is based on the exclusion of other causes of liver dysfunction, such as hepatitis A and B viruses, cytomegalo and Epstein–Barr virus infections, drug reactions including alcohol, autoimmune reaction, and metabolic liver diseases. The lack of a specific serological test for NANB hepatitis is an obstacle to preventing transmission of this virus.

Recently use of interleukin 2 (IL-2) has allowed the establishment of monoclonal T-cell lines (3, 4) and of T-cell clones that kill or recognize tumor cells or virus-infected cells (5, 6). Such T-cell clones have been used to identify a tumor-associated target antigen or to produce an antiidiotypic monoclonal antibody (mAb) of the internal image of a viral antigen that could be used as a vaccine (5, 7).

In this study we established a T-cell clone, termed TA-NB-2, cytotoxic for both autologous and allogeneic hepatocytes from patients with chronic NANB hepatitis. This clone may be useful as a tool to identify a NANB hepatitis-related antigen and develop an antigen–antibody system specific for NANB hepatitis. The development of an antiidiotypic mAb against TA-NB-2 clone that is the internal image of a NANB hepatitis-related target antigen may lead to its use as a vaccine for NANB hepatitis infection.

MATERIALS AND METHODS

Reagents and Media. Phytohemagglutinin-P and mitomycin C were obtained from Difco and Kyowa Hakko Kogyo (Tokyo), respectively. Fetal calf serum (FCS) and human IL-2 (lectin-free, 200 units/ml) were purchased from Boehringer Mannheim. Anti-CD3 (Leu-4), anti-CD4 (Leu-3a), anti-CD8 (Leu-2a), anti-CD11b (Leu-15), anti-CD16 (Leu-11b), and anti-Leu-19 mAbs, and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins were obtained from Becton Dickinson. WT31 mAb that recognized the common determinant of the α and β subunits of human T-cell receptor (TCR) was obtained from Sanbio BV-Biological Products (Heinspergenstraat, The Netherlands). Anti-human Ti-γA mAb, which was specific for the γ subunit of TCR, was a gift of Thierry Hercend (Institut Gustave-Roussy, Villejuif, France) (8). Anti-human TCR δ1 mAb that was specific for the δ subunit of TCR was a gift of Michael B. Brenner (Harvard University Medical School) (9). Anti-HLA class I and anti-HLA class II mAbs were purchased from Cosmo Bio (Tokyo). Basal medium consisted of MEM-α supplemented with glutamine (2 mM), pyruvate (1 mM), streptomycin (100 μg/ml), penicillin G (100 units/ml), anti-pleuropneumonia-like organism agent (Tylocine, 60 μg/ml; GIBCO), Hepes (15

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Abbreviations: NANB, type non-A and non-B virus; NANB hepatitis, NANB-associated hepatitis; IL-2, interleukin 2; FCS, fetal calf serum; TCR, T-cell receptor; CM, complete medium; PBL, peripheral blood lymphocytes; MHC, major histocompatibility complex; mAb, monoclonal antibody.

mM), and 2-mercaptoethanol (5×10^{-5} M). Complete medium (CM) consisted of RPMI 1640 medium supplemented with streptomycin (250 μg/ml), penicillin G (250 units/ml), Hepes (25 mM), and 10% FCS. IL-2 medium was basal medium supplemented with 20% FCS and IL-2 at 20 units/ml.

Separation of Human Peripheral Blood Lymphocytes. Human peripheral blood lymphocytes (PBL) were separated from heparinized peripheral blood using Ficoll-Paque (Pharmacia) gradient centrifugation.

Cloning by Limiting Dilution. Human T-cells were cloned from PBL obtained from a patient with chronic NANB hepatitis and a history of blood transfusion by the method described previously with some modifications (5). Briefly, PBL were suspended at a cell density of 5 cells per ml in IL-2 medium containing phytohemagglutinin-P at 10 μg/ml and mitomycin C-treated autologous PBL at 2×10^5 cells per ml. A 0.1-ml aliquot was placed in each well of the flat-bottomed 96-well microplate (Corning). The plate was incubated at 37°C in a humidified 5% CO_2 atmosphere, and 0.1 ml of IL-2 medium was added after 5-day incubation. After a 14-day culture period, cell growth was assessed microscopically, and the cells in any well where significant growth occurred were transferred into 24-well microplates (Corning). After reaching a density of 5×10^5 cells per ml, the cells were seeded into 25-cm^2 culture flasks (Corning) and were maintained at an initial concentration of 5×10^4 cells per ml in 10 ml of IL-2 medium. The medium was replaced every 3 days, and mitomycin C-treated autologous or allogeneic PBL and phytohemagglutinin-P were added every 10 days.

Microcytotoxicity Assay. The cytotoxic activity of cloned lymphocytes against human hepatocytes was assayed by a method described by Naumov et al. (10), because standard ^{51}Cr-release assay could not be used due to the small number of isolated hepatocytes obtained from the liver biopsy specimen and a high spontaneous release of ^{51}Cr from the hepatocytes. The cytotoxicity assay was done weekly with cloned lymphocytes obtained 6–8 days after stimulation with feeder cells and phytohemagglutinin-P. Briefly, 3–5 mm of the liver biopsy specimen from a patient was collected in a sterile tube containing CM gassed with 95% O_2/5% CO_2. The specimen was subsequently transferred to a small tissue-culture Petri dish containing CM supplemented with 5 mM EDTA and minced gently with two injection needles under sterile conditions. The resultant cell suspension was washed three times in CM gassed with 95% O_2/5% CO_2, followed by centrifugation at $50 \times g$ for 5 min. Ten microliters of cell suspension were seeded into each well of a microcytotoxicity Terasaki plate (163118; Nunc) to achieve a final concentration of ≈100 cells per well and were incubated at 37°C in a humid atmosphere of 95% O_2/5% CO_2. After 18- to 24-hr incubation of hepatocytes in the Terasaki plates, the supernatant was aspirated from each well, 1000 effector cells in 10 μl of IL-2 medium were added into each test well, and 10 μl of IL-2 medium alone was added into each control well. Ten replicate wells were prepared for each group.

After a further 18-hr incubation at 37°C, the plates were inverted for 1 hr to allow detachment of affected target cells and to clear the added effector cells and were gently washed with Hanks' balanced salt solution. Adherent hepatocytes were fixed with methanol for 5 min and stained with 1% aqueous eosin solution; then the number of adherent hepatocytes in each well was counted. Although only ≈30% of the seeded hepatocytes attached to the plates during the first 18- to 24-hr incubation, the number of the attached cells in each control well did not change during the next 18-hr incubation.

Preliminary studies showed that the detachment of hepatocytes from the plates was seen 4 hr after the start of the incubation with the effector cells, but the number of the detached hepatocytes was small at 4-hr incubation and increased during the next 14-hr incubation. The percentage of cytotoxic activity was calculated as [(mean no. of hepatocytes in control wells − mean no. of hepatocytes in test wells)/mean no. of hepatocytes in control wells] × 100. The cytotoxic activity of cloned cells against human hepatoma cells (SK-HEP-1, PLC/PRF/5) was also assessed by the microcytotoxicity assay as described above.

Inhibition of Cytotoxic Activity by mAbs. To investigate the effects of mAbs against the surface antigens of cloned cells, cloned cells at a density of 1×10^5 cells per ml were incubated with saturating amounts of each mAb for 30 min at 37°C in IL-2 medium. Subsequently, the cytotoxic activity of the cells was assessed by microcytotoxicity assay. The effects of anti-HLA mAbs were assessed by microcytotoxicity assay as follows. After 18- to 24-hr incubation of hepatocytes in the Terasaki plates, the hepatocytes in each well were incubated with saturating amounts of each anti-HLA mAb for 1 hr at 37°C, followed by incubation with effector cells for a further 24 hr at 37°C.

Immunofluorescence and Flow Cytometry. Cells (1×10^6) in 50 μl of basal medium with 10% FCS were incubated with the first-layer mAb on ice for 30 min. After washing with basal medium with 10% FCS, the cells were further incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins on ice for 20 min. The cells were washed with phosphate-buffered saline and fixed with 1% paraformaldehyde. Fixed cells were stored at 4°C in the dark until analysis. Single-color analysis was performed using a cell sorter (FACStar; Becton Dickinson).

Patients. Seventeen patients with chronic NANB hepatitis (11 males and 6 females) with a median age of 51 yr (range, 24–68 yr) and 12 control patients (9 males and 3 females) with a median age of 42 yr (range, 26–61 yr) were studied. All patients received peritoneoscopy and liver biopsy for diagnostic purpose. Diagnosis of NANB hepatitis was based on serological exclusion of hepatitis A and B, herpes, cytomegalo, and Epstein–Barr virus infections. Antinuclear, antimitochondrial, and smooth muscle antibodies were not detectible, and there was no history of drug- and alcohol-induced liver damage in patients with chronic NANB hepatitis. Fourteen patients had chronic hepatitis, and 3 patients had chronic hepatitis with cirrhosis. Eight of the 17 patients had a history of blood transfusion, and 2 of them had

Table 1. Cytotoxic activity of human T-cell clones against various target cells

	Target cells,* %				
	NANB hepatocytes		HBsAg-positive		
Effector	Autologous	Allogeneic	hepatocytes	SK-HEP-1	PLC/PRF/5
TA-NB-1	33.0	31.3	−9.7	−15.1	−3.3
TA-NB-2	46.2	51.4	−2.4	−1.8	−12.5

Target cells include hepatocytes from patients with chronic NANB hepatitis (NANB hepatocytes) and a patient with hepatitis B surface-antigen-positive chronic hepatitis (HBsAg-positive hepatocytes) and human hepatoma cells (SK-HEP-1, PLC/PRF/5)

*Percentage of cytotoxic activity at effector/target cell ratio of 10:1.

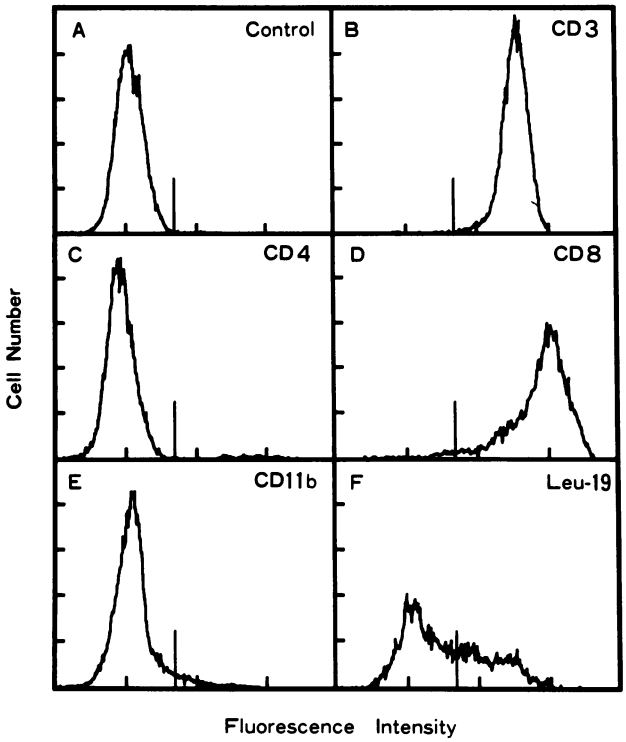

FIG. 1. Phenotype analysis of TA-NB-2 clone. TA-NB-2 cells were incubated with normal mouse IgG (control), anti-CD3, anti-CD4, anti-CD8, anti-CD11b, or anti-Leu-19 mAbs. Single-color analysis was performed by indirect immunofluorescence as described. Ordinate, relative cell number; abscissa, fluorescence intensity expressed in arbitrary logarithmic units.

known posttransfusion hepatitis. None of the 17 patients was positive for either hepatitis B surface antigen or hepatitis B core antibody. Control patients included 7 patients with hepatitis B surface-antigen-positive chronic hepatitis, 3 with

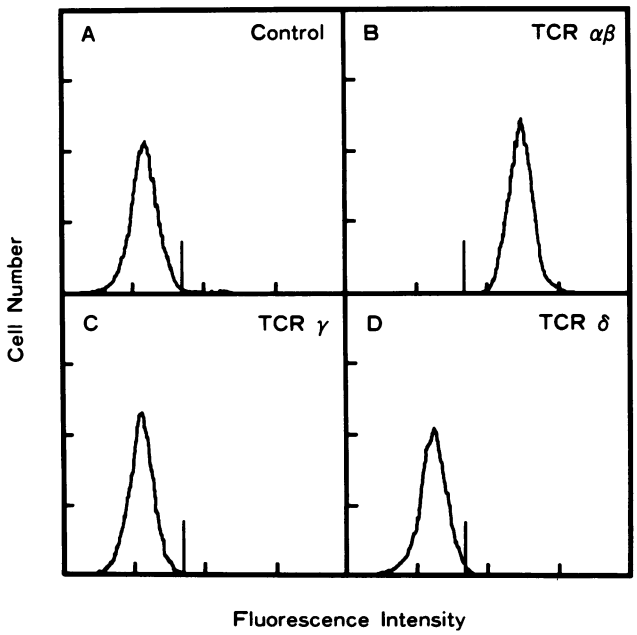

FIG. 2. Analysis of TCR subunits of TA-NB-2 clone. TA-NB-2 cells were incubated with normal mouse IgG (control), WT31, anti-Ti-γA, or anti-TCR δ1 mAbs. WT31 mAb recognizes TCR α and β heterodimer. Single-color analysis was performed by indirect immunofluorescence as described. Ordinate, relative cell number; abscissa, fluorescence intensity expressed in arbitrary logarithmic units.

Table 2. Effects of the treatment of TA-NB-2 clone with mAbs on the cytotoxic activity against hepatocytes from a patient with chronic NANB hepatitis

Treatment	Cytotoxicity,* %
Control (anti-CD4)	24.5 ± 6.5
Anti-CD3	−3.8 ± 6.5†
Anti-CD8	29.1 ± 1.8

*Mean ± SE for determination in triplicate of percentage of cytotoxic activity at effector/target cell ratio of 10:1.
†$P < 0.05$ compared with control.

acute hepatitis B, 1 with acute hepatitis A, and 1 with alcoholic liver cirrhosis. None of the control patients had known exposure to blood or blood products.

RESULTS

Cloning of Cytotoxic Cells Recognizing Hepatocytes from Patients with Chronic NANB Hepatitis. PBL from a patient with chronic NANB hepatitis and a history of blood transfusion were cloned by limiting dilution. Two clones (TA-NB-1, TA-NB-2), which showed cytotoxic activity against autologous hepatocytes, were obtained from 480 wells. As shown in Table 1, both clones lysed allogeneic hepatocytes from a patient with chronic NANB hepatitis as well as autologous hepatocytes, but neither hepatocytes from a patient with chronic type B hepatitis nor human hepatoma cell lines (SK-HEP-1, PLC/PRF/5). TA-NB-2 clone was chosen for the subsequent studies, because the growth of TA-NB-2 cells was better than that of TA-NB-1 cells. TA-NB-2 clone has been maintained for >1 yr without any loss of its function and cell-surface markers.

Cell-Surface Characterization of TA-NB-2 Clone. As shown in Fig. 1, >95% of TA-NB-2 cells expressed CD3 and CD8 antigens, but neither expressed CD4 nor CD11b antigens. Leu-19 antigen was variably expressed on about half of the TA-NB-2 cells. CD16 antigen was not expressed on TA-NB-2 cells (data not shown). These results suggest that TA-NB-2 clone was derived from $CD3^+$ $Leu\text{-}19^+$ non-major histocompatibility complex (MHC)-restricted cytotoxic T cells initially present in the peripheral blood (11). TA-NB-2 cells expressed a common antigen specific for the α and β subunits of TCR but neither of the antigens specific for the γ and δ subunits of TCR (Fig. 2).

Influence of mAbs on Cytotoxic Activity of TA-NB-2 Clone. To examine whether the antigen-recognition structure of TA-NB-2 cells was linked to CD3 antigen, a series of experiments were performed. Table 2 shows that the cytotoxic activity of TA-NB-2 cells against hepatocytes from a patient with chronic NANB hepatitis was abrogated by incubation with anti-CD3 mAb. In contrast, anti-CD8 mAb did not influence the cytotoxic activity of TA-NB-2 clone. When the cytotoxic activity of TA-NB-2 cells against hepatocytes from a patient with chronic NANB hepatitis was assessed in the presence of anti-HLA mAbs, both anti-HLA class I and anti-HLA class II mAbs had no influence on the cytotoxic activity (Table 3). The results suggest that the antigen-recognition structure of TA-NB-2 clone is function-

Table 3. Effects of anti-HLA mAbs on the cytotoxic activity of TA-NB-2 clone against hepatocytes from a patient with chronic NANB hepatitis

Treatment	Cytotoxicity,* %
Normal mouse IgG	43.8 ± 7.2
Anti-HLA class I	52.3 ± 4.5
Anti-HLA class II	59.5 ± 7.3

*Mean ± SE for determination in triplicate of percentage of cytotoxic activity at effector/target cell ratio of 10:1.

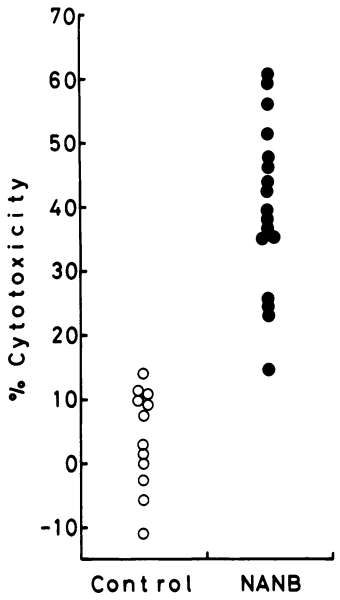

FIG. 3. Cytotoxicity of TA-NB-2 clone against hepatocytes from patients with chronic NANB hepatitis (NANB) and control patients (Control). The cytotoxic activity of TA-NB-2 cells was assessed by the microcytotoxicity assay described in text.

ally linked to CD3 antigen on the cell surface of the clone and that the cytotoxicity is non-MHC-restricted.

Cytotoxicity of TA-NB-2 Clone Against Hepatocytes from Patients with Chronic NANB Hepatitis. Specificity of the cytotoxicity of TA-NB-2 clone was evaluated using as targets hepatocytes from 17 patients with chronic NANB hepatitis and 12 control patients with liver disease unrelated to NANB hepatitis. Fig. 3 shows that the cytotoxic activity of TA-NB-2 clone against hepatocytes from patients with chronic NANB hepatitis was 39.8 ± 13.2% (mean ± SD) and ranged from 14.2 to 60.5%. In contrast, the cytotoxic activity of TA-NB-2 clone against hepatocytes from control patients was 4.0 ± 7.7% and ranged from −10.8 to 14.4%. The results suggest that TA-NB-2 cells specifically recognize and lyse NANB hepatitis virus-infected hepatocytes and that one agent is responsible for most, if not all, cases of chronic NANB hepatitis.

DISCUSSION

The present study was undertaken to establish a human cytotoxic T-cell clone that specifically recognized and lysed hepatocytes from patients with blood-borne NANB hepatitis. PBL from a patient with chronic NANB hepatitis and a history of blood transfusion were cloned by limiting dilution, and a T-cell clone, termed TA-NB-2, cytotoxic for both autologous and allogeneic hepatocytes from patients with chronic NANB hepatitis was established. TA-NB-2 clone belonged to $CD3^+$ $CD8^+$ non-MHC-restricted cytotoxic T cells and expressed an antigen specific for TCR α and β heterodimer. The cytotoxic activity of TA-NB-2 cells was abrogated by incubation with anti-CD3 mAb, but anti-HLA mAbs had no influence on the cytotoxicity of TA-NB-2 cells. Recent studies have shown that TCR is noncovalently associated with CD3 antigen on the cell surface of T cells (12). The association of TCR/CD3 complex on the cell surface of cytotoxic T cells has been shown by comodulation and loss of cytotoxic activity in response to anti-TCR or anti-CD3 mAbs (13). Taken together, TA-NB-2 cells recognize hepatocytes from patients with chronic NANB hepatitis by TCR in non-MHC-restricted manner.

When the cytotoxic activity of TA-NB-2 cells was assessed using as targets hepatocytes from patients with chronic NANB hepatitis and other patients with liver disease unrelated to NANB hepatitis, TA-NB-2 cells specifically recognized and lysed hepatocytes from patients with chronic NANB hepatitis. The patients with chronic NANB hepatitis included eight patients with a history of blood transfusion and nine patients without known exposure to blood or blood products. Two of the former eight patients had known posttransfusion hepatitis. It has been reported that cytotoxic T cells against virus-infected cells most often recognize the nucleoprotein of the virus expressed on the infected cells and lyse the cells (14). In addition, cytotoxic T cells have been suggested (*i*) to be responsible for the pathogenesis of chronic type-B hepatitis and (*ii*) to lyse hepatitis B-virus-infected hepatocytes by recognizing a hepatitis B core antigen expressed on the infected cells (10, 15). Taken together, TA-NB-2 cells quite possibly lyse NANB hepatitis virus-infected hepatocytes by recognizing a NANB hepatitis virus-associated nucleoprotein expressed on the infected cells. Thus, one agent would be responsible for most, if not all, cases of blood-borne NANB hepatitis. The latter idea is opposed to the hypothesis that at least two blood-borne NANB hepatitides exist. The latter hypothesis is based on the findings that the second episodes of NANB hepatitis can occur in man and in experimentally infected chimpanzees (16–18). Brotman *et al.* (19), however, recently provided evidence against the hypothesis that so-called second bouts of NANB hepatitis can be induced by rechallenge with the same inoculum.

Antiidiotypic antibody against an antigen-binding site of TCR has been reported to be the internal image of the target antigen and to show complete cross-reaction with relevant idiotypic B-cell receptor (14, 20). TA-NB-2 cells most probably recognize a NANB hepatitis virus-associated nucleoprotein expressed on the infected hepatocytes, and the recognition is not restricted by the MHC antigens. Thus anti-idiotypic antibody against the antigen-binding site of TCR of TA-NB-2 cells may be the internal image of a NANB hepatitis virus-associated antigen, and this antigen may be able to be used as a surrogate for a NANB hepatitis-related antigen in the serological diagnosis of NANB hepatitis. Such an anti-idiotypic antibody might also be used as a vaccine for a NANB hepatitis-virus infection, as proved in a Sendai virus infection in mice (7).

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