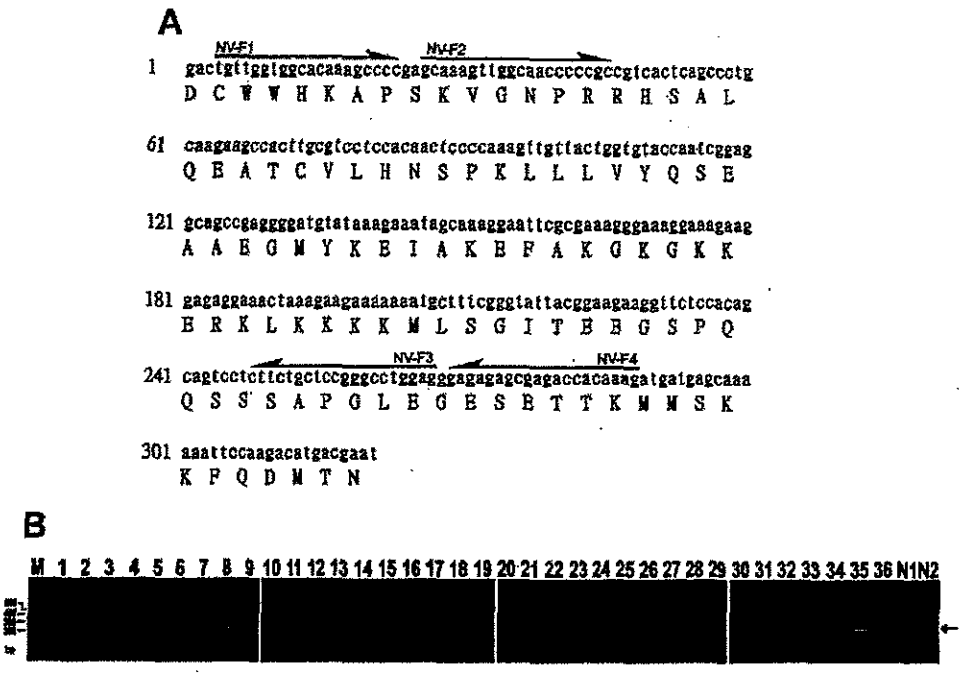

Figure 1. Identification of a foreign sequence in patients with non–A–E hepatitis. *A*, Nucleotide sequence of the NV-F DNA fragment and conceptual translation of the putative partial reading frame. The positions of 4 primers (NV-F1 to NV-F4) used for polymerase chain reaction (PCR) detection of NV-F are marked with arrows. *B*, Serum samples from patients with non–A–E hepatitis *(lanes 1–9)*, patients infected with hepatitis C virus *(lanes 10–19)*, patients infected with hepatitis B virus *(lanes 20–29)*, and healthy individuals *(lanes 30–36)* subjected to an NV-F detection assay. Only part of the results is shown here. M, molecular weight marker; N1, negative control (NV-F–negative serum sample); N2, negative control (pure water). The arrow indicates the PCR product of NV-F.

PATIENTS, MATERIALS, AND METHODS

Patients and samples. After informed consent was obtained, the remaining aliquots of serum samples submitted for biochemical tests in patients visiting Chang Gung Medical Center were collected for this study. Samples from 4 groups of patients were included for NV-F sequence detection: (1) 180 healthy subjects (from Health Examination Service, Chang Gung Medical Center) with normal alanine aminotansferase (ALT) levels who were negative for HBV surface antigen (HBsAg), anti-HCV antibody, and HEV RNA; (2) 150 patients with hepatitis B who were positive for HBsAg and negative for IgM class anti-HAV antibody, anti-HDV antibody, anti-HCV antibody, and HEV RNA; (3) 150 patients with hepatitis C who were negative for HBsAg and IgM anti-HAV antibody, positive for anti-HCV antibody, and negative for HEV RNA; and (4) 69 patients with non–A–E hepatitis with serum ALT levels elevated >2.5-fold who were negative for HBsAg, IgM anti-HAV antibody, IgM class antibody against HBV core antigen (HBc), anti-HCV antibody, HEV RNA, and HCV RNA. None of these patients were alcoholics, and no known hepatotoxic medicine had been taken. Patients with fatty liver were not excluded from this study. All patients were negative for autoimmune markers, including antinuclear antigen, anti–smooth muscle antigen, and anti-mitochondrial antigen. In addition, patients were all negative for other virological markers, including antibody for HIV, IgM class antibody for Epstein-Barr virus, and IgM class antibody for cytomegalovirus. After the polymerase chain reaction (PCR) assays for the NV-F sequence, adequate amounts of samples were still available for the detection of anti–NV-F antibody in 155 patients. After informed consent was obtained, liver biopsy samples from 2 patients (patients F and B) whose serum was positive for the NV-F sequence were subjected to immunofluorescence analysis.

Serological studies. HBsAg, IgM anti-HAV antibody, IgM anti-HBc antibody, and anti-HDV antibody were assayed using radioimmunoassay kits (*Ausria-II*, *HAVAB-M*, and *anti-delta*; Abbott Laboratories). Anti-HCV antibody was detected using an enzyme immunoassay kit (HCV-II; Abbott Laboratories). HCV RNA was detected by reverse transcription (RT) PCR assay (Amplicor HCV test; Roche Diagnostic Systems). HBV DNA was detected by Amplicor HBV Monitor Test (Roche Molecular Systems). The method of HEV RNA detection has been described elsewhere [3, 4].

Extraction of DNA or RNA, RT-PCR, and PCR. Total serum DNA was extracted using proteinase K digestion followed by phenol/chloroform extraction, as described in our previous publication [11]. Total serum RNA was extracted using TRI

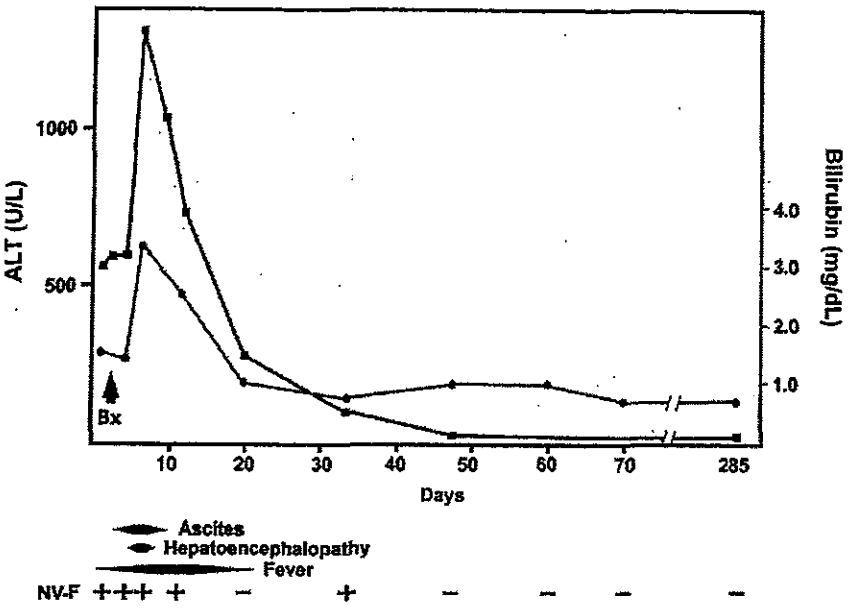

Figure 2. Clinical course in a patient with fulminant non-A–E hepatitis. Squares denote alanine aminotansferase (ALT) levels (U/L), and circles denote bilirubin levels (mg/dL). The periods of clinical symptoms are marked with solid bars, and "Bx" indicates the time of the liver biopsy. The NV-F sequence was detected by polymerase chain reaction in serial serum samples, and the results are indicated by a plus or a minus symbol.

reagent (Molecular Research Center), in accordance with the protocol provided by the manufacturer. RT was performed using random primers. The procedure for RT and PCR has been described elsewhere [12]. Three primers were engineered: P1, 5′-CCGCGG$(N)_4$-3′; P2, 5′-GAATTC$(N)_4$-3′; and P3, 5′-GCTTGCTCTGTCTC$(T)_{20}$-3′. Each of the 4 Ns in P1 and P2 was a mixture of A, T, C, and G in equal ratios. After extraction of the total serum DNA or RNA from patient L, PCR or RT-PCR was performed, using random hexamers for 25 cycles; the product was then amplified using any 2 of the P1–3 primers. The resulting products were cloned into a vector, pCR2.1-TOPO (Invitrogen). For PCR detection of *Escherichia coli* 16S ribosomal DNA, the following primers were used: 16SL, 5′-GTCTGGGAAACTGCCTGATG-3′ (nt 121–140) and 16SR, 5′-GCTTCTTCTGCGGGTAACGT-3′ (nt 500–481).

Elimination of clones derived from the human genome. To eliminate clones derived from the human genome, the clones were first lifted onto a nitrocellulose filter and hybridized with a mixture of probes generated from total liver RNA, as described in our previous publication [13]. Briefly, single-stranded probe was generated from cytoplasmic RNA extracted from normal human liver tissue. The tissue was minced into small pieces and lysed in a buffer containing 10 mmol/L Tris HCl (pH 7.2), 150 mmol/L NaCl, and 0.5% Nonidet P-40 (Sigma). After centrifugation at 1500 *g* for 5 min, the supernatant was used for RNA extraction. RT was performed using SuperScript II RNase H minus Reverse Transcriptase (Invitrogen), and oligo(dT) was used as the RT primer. One-third of the dTTP in the dNTP mixture was replaced by digoxigenin-11-dUTP (Boehringer Mannheim) to generate digoxigenin-labeled probes. The probes were mixed (molar ratio, 1:2) with oligo(dA) at 40°C for 1 h before hybridization. The hybridization signal was detected by use of a DIG Luminescent Detection Kit (Boehringer Mannheim). For each batch of hybridization, 1 ng of pCR2.1-TOPO without a cDNA insert was used as a negative control, and 1 pg of pCR2.1-TOPO containing a fragment of human albumin gene (Hs.184411) was used as a positive control. The negatively hybridized clones were considered to be of nonhuman origin.

Automatic sequencing. The nonhuman-origin clones were subjected to automatic DNA sequencing (CEQ 2000; Beckman Instruments). The sequence data were further searched against the National Center for Biotechnology Information (NCBI) human genome data bank (http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html), to eliminate human sequence.

Development of anti–NV-F antibody. The putative partial coding sequence of NV-F, flanked by NV-F1 and NV-F4 primers, was inserted into a vector, pYES2/NT (Invitrogen Corporation), and was arranged in-frame with the upstream polyhistidine region and the Xpress epitope sequence. The coding region of the whole fusion protein was subsequently isolated by restriction enzyme digestion (*Hin*dIII to *Xba*I), blunt-ended, and inserted into the *Sma*I site of pBacPAK8 (Clontech Laboratories). The fusion protein was expressed using the BacPak Baculovirus Expression System (Clontech). It was purified by a Ni^{2+}-charged

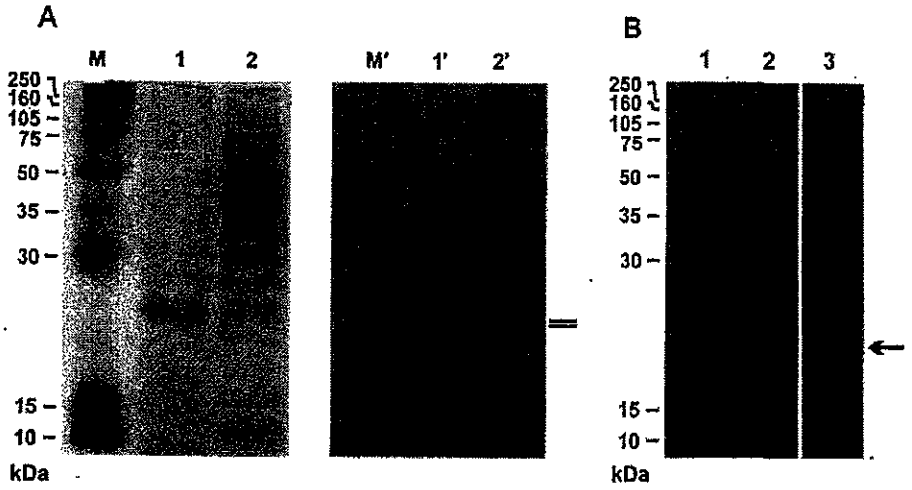

Figure 3. Generation of NV-F peptide and development of antibody against NV-F antigen. *A*, A fusion protein containing polyhistidine, Xpress epitope, and a peptide encoded by NV-F was expressed in insect cells. The protein extract was purified by affinity column and was analyzed by electrophoresis. The molecular weight marker (*M* and *M'*), purified protein (*lanes 1* and *1'*), and nonpurified cell lysate (*lanes 2* and *2'*) were visualized by either coomassie blue staining (*M*, *lanes 1* and *2*) or Western blot analysis using anti-Xpress antibody (*M'*, *lanes 1'* and *2'*). The purified protein was then used to develop a mouse polyclonal antibody against NV-F. *B*, The NV-F peptide alone (no fusion parts), subsequently expressed in insect cells. The cell lysate containing NV-F peptide *(lane 1)* and a mock control *(lane 2)* were analyzed by Western blot using the mouse anti–NV-F antibody. The cell lysate containing NV-F peptide was also analyzed, using a patient's serum that was positive for the NV-F sequence *(lane 3)*.

affinity column and was injected into a mouse for development of a polyclonal antibody. Alternatively, an initiation codon (ATG) was engineered in-frame with the putative coding sequence, and the resulting sequence was inserted into pBacPAK8, to express an NV-F peptide that did not contain any fusion parts. The primer used to generate the initiation codon (underlined) was 5′-ATGTGTTGGTGGCACAAAGCCC-3′.

Immunofluorescence analysis. Fragments of liver specimens were snap frozen in isopentane cooled with liquid nitrogen and were stored at −70°C until use. Cryostat sections (5 μm) were dried at room temperature overnight and fixed in acetone at 0°C for 5 min. The immunofluorescence staining was performed using mouse polyclonal antibody against NV-F followed by fluorescein isothiocyanate–conjugated rabbit anti-mouse antibody (Jackson Immuno Research Laboratories). Double staining was performed by simultaneously staining the nuclei with DAPI (200 ng/mL). Confocal microscopy was performed using a Leica TCS SP2 Laser Scanning Spectral Confocal System.

RESULTS

Strategy to identify foreign sequences in the serum sample of a patient with non–A–E hepatitis. A 66-year-old man (patient L) received a diagnosis of colon cancer (adenocarcinoma in transverse colon) in December 1999 at Chang Gung Medical Center. He received a colectomy, which was later complicated by anastomosis leakage, sepsis, and gastric ulcer bleeding. After intensive medical treatment, including blood transfusion, the patient's condition was gradually stabilized. Unfortunately, an episode of acute hepatitis (peak ALT level, 284 U/L) with deep jaundice (bilirubin level, 19 mg/dL) occurred in July 2000. The patient was found to be negative for HBsAg, IgM anti-HAV antibody, IgM anti-HBc antibody, anti-HDV antibody, and anti-HCV antibody. The patient also tested negative for HEV RNA and HCV RNA. The serum sample obtained at this point was used for molecular cloning of foreign sequences.

To identify foreign sequences in the serum sample, total serum DNA or RNA was extracted. The nucleic acid was then amplified (by PCR or RT-PCR) using random primers. The amplified product was subsequently subjected to a second-step PCR using designed primers (see Patients, Materials, and Methods). To eliminate sequence derived from human chromosomes, the resulting clones were hybridized with the probes generated from cytoplasmic RNA of normal liver tissue. All positively hybridized clones were discarded. The remaining 195 clones were sequenced using an automatic DNA sequencer. The sequencing data were compared with the human genome sequence, as well as with sequences in GenBank, by use of NCBI BLAST. Only 3 clones were found to be of nonhuman origin. One of the sequences, derived from the DNA extract, contained an open reading frame with incomplete 5′ and 3′ ends and was temporarily named NV-F (figure 1*A*). The sequence potentially encoded a peptide with incomplete amino- and carboxy-termini. Four primers, NV-F1 to NV-F4, were designed for the nested PCR assay. By use of this assay, this sequence was found to be absent in the chromosomal DNA extracted from HepG2 cells, Daudi cells, and 3 different sources of human peripheral blood mononuclear cells.

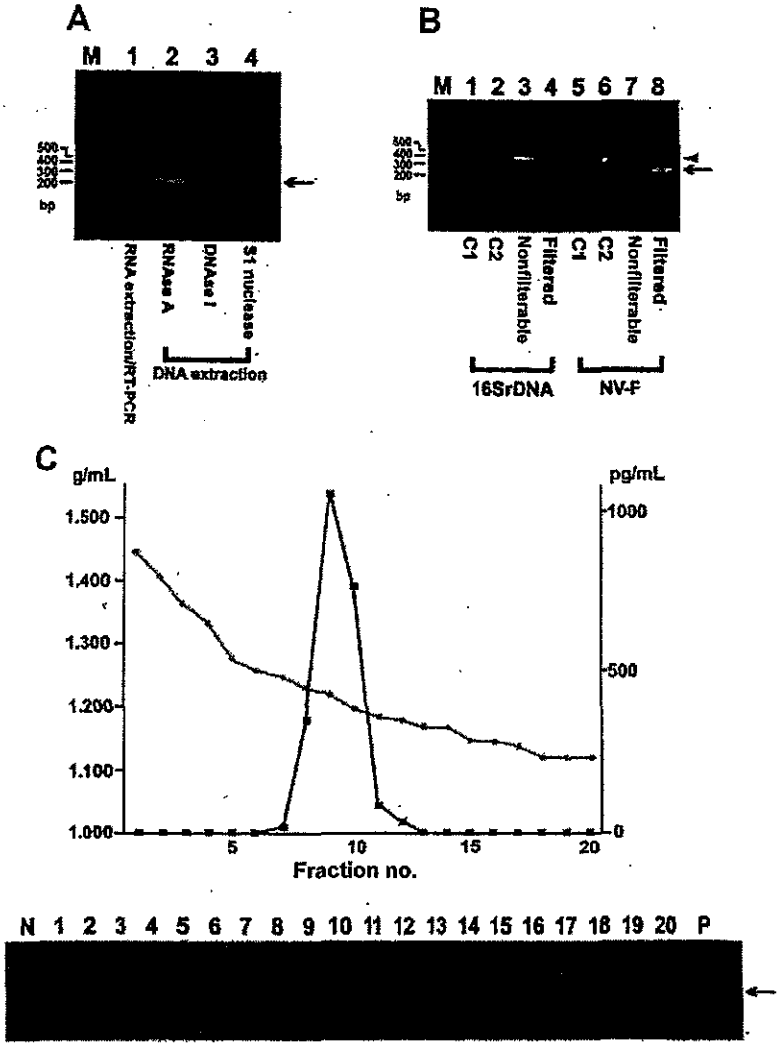

Figure 4. Characterization of the NV-F agent. *A,* Extraction of nucleic acid from the serum sample by either the RNA extraction *(lane 1)* or the DNA extraction method *(lanes 2–4).* After RNA extraction, reverse transcription (RT) polymerase chain reaction (PCR) for detection of the NV-F sequence was performed, without any intermediate step *(lane 1).* After DNA extraction, the extracted sample was treated with RNAse A *(lane 2),* DNAse I *(lane 3),* or S1 nuclease *(lane 4)* before subsequent PCR assay for NV-F sequence. M, molecular weight marker. The arrow indicates the PCR product of NV-F. *B,* Size assessment of the NV-F agent. Serum containing the NV-F agent was mixed with *Escherichia coli* and passed through a filter with a pore size of 0.2 μm. PCR was performed to detect 16S ribosomal DNA of *E. coli (lanes 1–4)* or NV-F *(lanes 5–8)* in filtered (*lanes 2, 4, 6,* and *8*) or nonfilterable (*lanes 1, 3, 5,* and *7*) fractions. An aliquot of serum negative for the NV-F sequence (C1 and C2) was assayed in parallel as a mock control. The arrowhead indicates the PCR product of 16S ribosomal DNA, and the arrow indicates the PCR product of NV-F. *C,* Cesium chloride gradient analysis for the NV-F agent. A serum sample positive for both hepatitis B virus (HBV) DNA and the NV-F sequence was used for cesium chloride gradient analysis. Twenty fractions were collected. All were sent for both HBV DNA quantitation *(upper panel)* and 1-step PCR (for the NV-F sequence) followed by Southern blot analysis *(lower panel).* Circles denote densities, and squares denote HBV DNA levels. The arrow indicates the PCR product of the NV-F sequence. N, negative hybridization control (1 ng of pCR2.1-TOPO); P, positive hybridization control (1 ng of the NV-F sequence).

Detection of the NV-F sequence in patients with non–A–E hepatitis. Serum samples from 4 groups of patients were included for the detection of the NV-F sequence (figure 1*B*). The sequence was detected in 5 (2.8%) of 180 healthy individuals. In contrast, NV-F was present in 17 (24.6%) of 69, 21 (14.0%) of 150, and 42 (28%) of 150 patients with non–A–E hepatitis, chronic hepatitis B, and chronic hepatitis C, respectively. One of the 17 patients whose serum was positive for NV-F had fulminant hepatitis. This was a 47-year-old male (patient F) who had non–A–E hepatitis accompanied by intermittent high fever and chills in May 2003. He was admitted for liver biopsy and further clinical investigation. Liver decompensation with

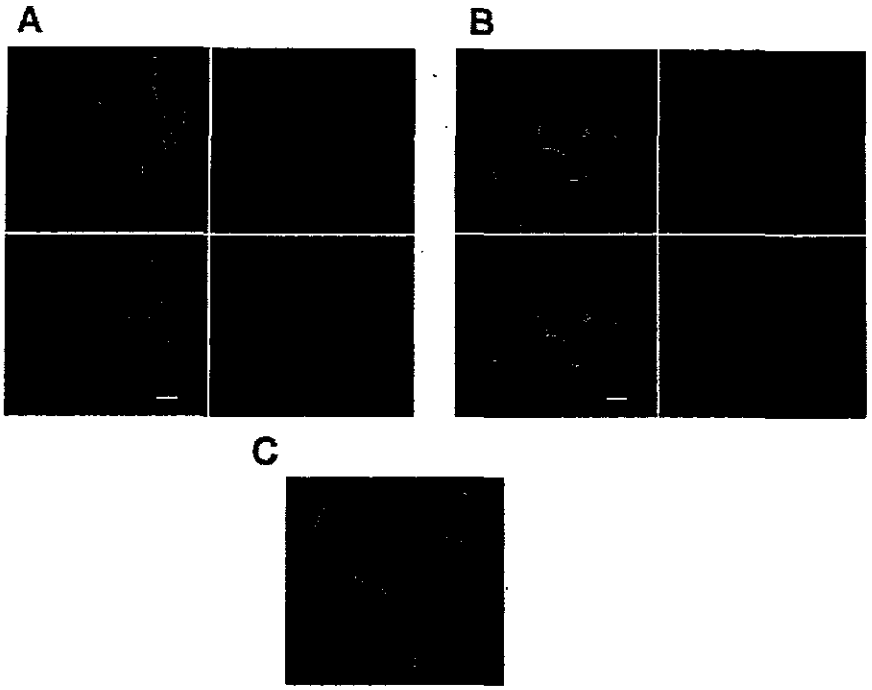

Figure 5. Detection of NV-F antigen in the liver biopsy sample from a patient with fulminant non–A–E hepatitis (patient F). Two different sections (*A* and *B*) from the same biopsy are shown. Positive cells in panel B are shown at higher magnification in panel C. Immunofluorescence analysis was performed using anti–NV-F antibody *(left upper panel)* and DAPI *(right upper panel)* for double staining. The pictures were overlapped using confocal microscopy *(left lower panel)*. A negative control *(right lower panel)* using preimmune serum for staining was included. Scale bar, 20 μm.

massive ascites, bilateral pleural effusion, and consciousness disturbance developed 10 days after onset. Thereafter, the patient's condition improved progressively without the need for any specific treatment, and he finally recovered completely. No known infectious agent was found throughout the course of the illness. Serial serum samples were obtained from this patient; his serum was found to be positive for the NV-F sequence during the early stage of the hepatitis flare, but it became negative thereafter (figure 2).

Expression of NV-F peptide and detection of anti–NV-F antibody. The putative coding sequence flanked by NV-F1 and NV-F4 was used to express a fusion protein containing the putative NV-F antigen, polyhistidine, and an Xpress epitope, using insect cells. After purification, a doublet was found in the protein gel, which could also be seen by Western blot using anti-Xpress antibody (figure 3*A*). A mouse polyclonal antibody was then raised against the fusion protein. This antibody recognized a single protein species when only the NV-F peptide (no other fusion parts) was expressed in the insect cells (figure 3*B*, *lane 1*). By use of this peptide as an antigen, anti–NV-F antibody in serum from patient L was assayed. Western blot analysis revealed only 1 protein species (figure 3*B*, *lane 3*). The doublet derived from the fusion protein was, therefore, likely a result of partial degradation. Serum samples were subsequently examined for the presence of anti–NV-F antibody, using the insect cell lysate containing NV-F peptide (no other fusion parts) as well as the purified NV-F fusion protein as an antigen. The results obtained by use of the 2 methods were consistent. It was found that anti–NV-F antibody was present in 49 (75.4%) of the 65 patients whose serum was found to be positive for the NV-F sequence, including patient L and patient F. Of the 49 positive samples, 15 were from patients with non–A–E hepatitis, 16 were from those with chronic hepatitis B, and 18 were from those with chronic hepatitis C. In contrast, anti–NV-F antibody was undetectable in 90 patients whose serum was negative for the NV-F sequence (49 healthy individuals, 10 patients with non–A–E hepatitis, 11 patients with chronic hepatitis B, and 20 patients with chronic hepatitis C).

Characterization of the NV-F–associated agent. The nucleic acid was extracted from the serum sample from patient L, using either a DNA or an RNA extraction method. The nucleic acid was then digested by DNAse I, RNAse A, or S1 nuclease before the PCR assay. The results showed that the NV-F sequence was present only in the nucleic acid fraction that was extracted using the DNA extraction method. The NV-F sequence was resistant to RNAse A digestion but was sensitive to DNAse I and S1 nuclease digestion (figure 4*A*). To estimate the size of the NV-F–associated agent, the serum sample was mixed with 10^5 *E. coli*

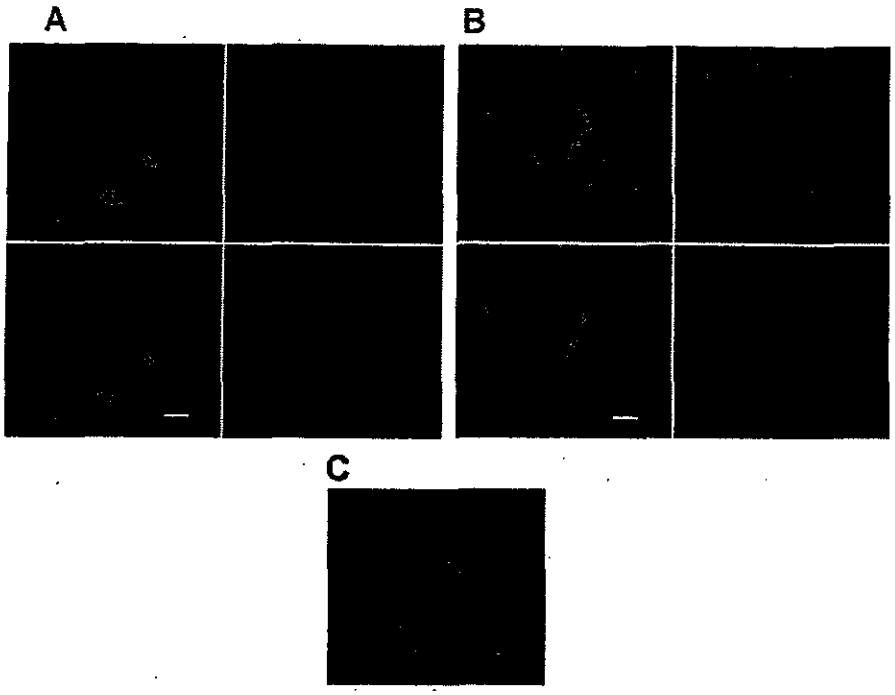

Figure 6. Detection of NV-F antigen in the liver biopsy sample from a patient with NV-F and hepatitis B virus coinfection (patient B). See the legend to figure 5 for further details.

organisms and passed through a filter with a pore size of 0.2 μm. The nonfilterable material was resuspended in PBS and analyzed in parallel with the filtered portion. The result indicated that the putative particles containing the NV-F sequence were smaller than 0.2 μm (figure 4*B*).

It was found that, in some patients with chronic hepatitis B, coinfection with the NV-F agent and HBV occurred. A 36-year-old male (patient B from the chronic hepatitis B group) who had chronic hepatitis B with mild activity for >2 years came to our clinic to undergo a liver biopsy for fibrosis staging. A PCR assay revealed that his serum was also positive for NV-F. The serum sample from patient B was subjected to cesium chloride gradient analysis. The gradients were fractionated and assayed for the presence of HBV DNA (using a quantitative test) and the NV-F sequence (using 1-round PCR followed by Southern blot analysis). Two peaks of NV-F sequence were present, one in the fractions of 1.33–1.39 g/mL and the other in the fractions of 1.22–1.25 g/mL (figure 4*C*). The peak HBV DNA concentration was found in the fraction of 1.19–1.21 g/mL, indicating that the HBV particles were slightly lighter than the NV-F–associated particles. This experiment was repeated using serum samples from 3 other patients with NV-F–associated hepatitis, and the results were consistent.

Immunofluorescence analysis. By use of the NV-F fusion protein expressed in insect cells, mouse anti–NV-F antibody was developed for immunofluorescence analysis. This antibody specifically detected the putative NV-F antigen (figure 3*B*). Immunofluorescence analysis was performed on the liver biopsy tissue obtained from patient F (figure 5) and patient B (figure 6). It was found that the antigen was distributed either in a speckle pattern or homogeneously in the cytoplasm of hepatocytes. Furthermore, positive staining was also observed in the perinuclear area (or on the nuclear membrane) in most positively stained cells.

DISCUSSION

Owing to technological advances in molecular biology, 5 major hepatitis viruses (HAV to HEV) have been discovered. The etiology of chronic hepatitis can thus be determined in a great majority of patients. Despite this achievement, the cause of chronic hepatitis remains elusive in ~5% of patients [5, 14]. Furthermore, in acute hepatitis, the proportion of patients with undetermined etiology is even higher [3, 15]. In Taiwan, the HBV carrier rate is ~15%, and more than half of the inpatients in Taiwan with acute hepatitis are seropositive for HBsAg [3]. It is believed that acute exacerbation of hepatitis B in chronic HBV carriers is responsible for the majority of acute hepatitis flares [16]. Even though the proportion of patients with non-B hepatitis is small, the etiology of acute hepatitis remains undetermined in 15.9% of our inpatients, suggesting the existence of other, unidentified hepatitis viruses [3]. In this study, we have

identified a fragment of DNA sequence (NV-F) in the serum of a patient with non–A–E hepatitis. Only 2.8% of healthy individuals carried this sequence in their serum, whereas 24.6% of patients with non–A–E hepatitis were positive for NV-F. In this study, we did not exclude patients with nonalcoholic steatohepatitis from the non–A–E hepatitis group, nor did we exclude patients with fatty liver [17, 18]. It is possible that the prevalence of NV-F would be even higher if such patients were excluded. Interestingly, a high prevalence of NV-F is also observed in patients with chronic hepatitis B or C, indicating that coinfection with NV-F and either HBV or HCV frequently occurs. Similarly, when HCV was initially discovered, many studies on the seroprevalence of HCV indicated that HCV was found in >10% of HBV-infected patients worldwide [19]. The prevalence might be underestimated, since HCV superinfection exerts a suppressive effect on HBV and enhanced seroclearance of HBV [20]. Coinfection with HBV or HCV was also commonly found in patients with GBV-C, TTV, and SEN virus infection. Supposedly, such a high percentage of coinfection is attributed to a common transmission route. The effect of NV-F superinfection on chronic hepatitis B or C is not clear at this time. A detailed clinical analysis is needed to answer this question. Despite a high prevalence of the NV-F agent in non–A–E hepatitis, it is still questionable whether NV-F is the direct cause of hepatitis. Since NV-F frequently coinfects with HBV or HCV, it remains possible that NV-F coinfects with a yet-unidentified virus in patients with non–A–E hepatitis and that it is the unidentified virus that serves as the direct cause of hepatitis. In this study, we have provided 2 pieces of evidence suggesting that NV-F might contribute, at least in part, to the hepatitis activity. First, in patient F, NV-F viremia occurred concurrently with the hepatitis flare, and the NV-F agent was cleared from the serum after recovery from the disease. This temporal relationship argues for a causative role of NV-F in non–A–E hepatitis. Second, the NV-F antigen was found in the cytoplasm of hepatocytes, suggesting that this agent is hepatotropic. The presence of a foreign antigen in the liver cells frequently results in an inflammatory reaction—namely, hepatitis—unless other unknown mechanisms are involved to deter the host immune response. Further immunological study is needed to understand the mechanism of NV-F–associated non–A–E hepatitis.

At this time, the biological nature of the NV-F agent has not been completely defined. Our data indicate that it is smaller than 0.2 μm, forms 2 buoyant densities in a cesium chloride gradient, and possesses single-stranded DNA. These features suggest that the NV-F agent is possibly a virus. The presence of 2 densities in cesium chloride gradient analysis is sometimes observed in an enveloped virus. A possible explanation is that some particles containing only the nucleocapsid (but not the envelope) form the band with the higher density. However, owing to an extremely low serum concentration of NV-F, the attempt to visualize the particles by electron microscopy failed. Southern and Western blot analysis using the remaining liver biopsy samples submitted for this study from patients L and B (only 3 mm in length) failed to demonstrate the viral genome and protein. A larger piece of tissue, such as surgically removed liver tissue, may be required to achieve this goal. A BLAST search showed that none of the known sequences shared sequence homology with NV-F. Further extension of the 5′ and 3′ ends of the NV-F sequence is, thus, progressing very slowly. The best-known single-stranded DNA viruses are parvoviruses and circoviruses. It is possible that NV-F belongs to a class of virus distantly related to one of these 2 families. Alternatively, it may represent a new class of agents that has no known close relatives.

In summary, we have discovered a novel single-stranded DNA sequence that is associated with human hepatitis, including in a patient with fulminant non–A–E hepatitis. The NV-F agent is hepatotropic and likely belongs to a novel class of viruses. Finally, this virus frequently coinfects with HBV or HCV in patients with chronic hepatitis.

References

1. Dienstag JL, Alter HJ. Non-A, non-B hepatitis: evolving epidemiologic and clinical perspective. Semin Liver Dis 1986; 6:67–81.
2. Reyes GR, Baroudy BM. Molecular biology of non-A, non-B hepatitis agents: hepatitis C and hepatitis E viruses. Adv Virus Res 1991; 40: 57–102.
3. Chu CM, Lin SM, Hsieh SY, et al. Etiology of sporadic acute viral hepatitis in Taiwan: the role of hepatitis C virus, hepatitis E virus and GB virus-C/hepatitis G virus in an endemic area of hepatitis A and B. J Med Virol 1999; 58:154–9.
4. Chu CM, Sheen IS, Liaw YF. The role of hepatitis C virus in fulminant viral hepatitis in an area with endemic hepatitis A and B. Gastroenterology 1994; 107:189–95.
5. Kodali VP, Gordon SC, Silverman AL, McCray DG. Cryptogenic liver disease in the United States: further evidence for non-A, non-B, and non-C hepatitis. Am J Gastroenterol 1994; 89:1836–9.
6. Romeo R, Pol S, Demeret C, et al. Evidence of non-A, non-B, non-C infection in chronic hepatitis by polymerase chain reaction testing for hepatitis B and C viruses. J Hepatol 1995; 22:125–9.
7. Van Thiel DH, Gavaler JS, Baddour N, Friedlander L, Wright HI. Treatment of putative non-A, non-B, non-C hepatitis with alpha interferon: a preliminary trial. J Okla State Med Assoc 1994; 87:364–8.
8. Stapleton JT. GB virus type C/hepatitis G virus. Semin Liver Dis 2003; 23:137–48.
9. Cossart Y. TTV—a virus searching for a disease. J Clin Virol 2000; 17: 1–3.
10. Akiba J, Umemura T, Alter HJ, Kojiro M, Tabor E. SEN virus: epidemiology and characteristics of a transfusion-transmitted virus. Transfusion 2005; 45:1084–8.
11. Hammel P, Marcellin P, Martinot-Peignoux M, et al. Etiology of chronic hepatitis in France: predominant role of hepatitis C virus. J Hepatol 1994; 21:618–23.
12. Thiers V, Lunel F, Valla D, et al. Post-transfusional anti-HCV-negative non-A non-B hepatitis (II) serological and polymerase chain reaction analysis for hepatitis C and hepatitis B viruses. J Hepatol 1993; 18:34–9.
13. Liaw YF, Yang SS, Chen TJ, Chu CM. Acute exacerbation in hepatitis

B e antigen positive chronic type B hepatitis: a clinicopathological study. J Hepatol 1985; 1:227–33.
14. Brunt EM. Nonalcoholic steatohepatitis. Semin Liver Dis 2004; 24:3–20.
15. Angulo P. Nonalcoholic fatty liver disease. N Engl J Med 2002; 346: 1221–31.
16. Liaw YR Role of hepatitis C virus in dual and triple hepatitis virus infection. Hepatology 1995; 22:1101–8.
17. Liaw YF, Chen YC, Sheen IS, Chien RN, Yeh CT, Chu CM. Impact of acute hepatitis C virus superinfection in patients with chronic hepatitis B virus infection. Gastroenterology 2004; 126:1024–9.
18. Yeh CT, Lu SC, Tseng IC, et al. Antisense overexpression of BMAL2 enhances cell proliferation. Oncogene 2003; 22:5306–14.
19. Yeh CT, Chien RN, Chu CM, Liaw YF. Clearance of the original hepatitis B virus YMDD-motif mutants with emergence of distinct lamivudine-resistant mutants during prolonged lamivudine therapy. Hepatology 2000; 31:1318–26.
20. Yeh CT, Lu SC, Chu CM, Liaw YF. Molecular cloning of a defective hepatitis C virus genome from the ascitic fluid of a patient with hepatocellular carcinoma. J Gen Virol 1997; 78:2761–70.

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研究報告の概要

○オランダにおける血小板輸血の臨床的安全性に関する手指消毒法、初流血除去バッグ、細菌スクリーニングの効果
背景:血液製剤の細菌汚染は、致死的な輸血副作用が発現する大きな危険性がある。好気的および嫌気的培養(BacT/ALERT, bioMerieux)による濃厚血小板(PC)の細菌スクリーニングは、2001年10月にオランダに導入された。
実験デザインおよび方法:2002年11月、70%イソプロピルアルコールのダブルスワブで消毒するという全国統一の皮膚消毒法が導入された。ある施設では、日常的に初流血除去バッグを使用して初流血20～30mLを除去していた。
結果:2002年から2003年にかけて、プールバフィーコート由来のPC合計113,093のスクリーニング検査が行われた。新しい消毒法の導入後、0.85%は初回陽性であった。初回陽性が0.95%であったこれまでの消毒法と比較して、減少幅は小さかった。初流血除去バッグを使用していた施設では、細菌汚染の頻度が有意に低く、70%イソプロピルアルコールによる消毒法の導入前は0.50%、導入後は0.37%であった。また、アフェレーシスPC8000件のスクリーニング検査も行われ、初回陽性は24検体(0.30%)であった。
結論:初流血除去バッグの使用および、減少幅は小さいものの70%イソプロピルアルコールのダブルスワブの使用によって、細菌汚染が減少した。予測された通り、大半を占める皮膚常在菌の汚染が減少した。初流血除去バッグと新たな消毒法を併用した場合、初回陽性の発現頻度は、供血者5名のプールPCと単一供血者由来のアフェレーシスPCと同程度のものであった。さらに、細菌検出システムとそれに伴う製剤回収手順は、特に急速に増殖する細菌によって汚染されたPCや赤血球の輸血による感染防止に効果的であることが示された。

報告企業の意見	今後の対応
初流血除去バッグの使用および70%イソプロピルアルコールのダブルスワブの使用によって、輸血用血液製剤の細菌汚染が減少したとの報告である。	日本赤十字社では、「血液製剤等に係る遡及調査ガイドライン」(平成17年3月10日付薬食発第0310009号)における「本ガイドライン対象以外の病原体の取扱い イ. 細菌」に準じ細菌感染が疑われる場合の対応を医療機関に周知している。 今後も情報の収集に努める。採血時の初流血除去、白血球除去の導入とともに細菌を不活化する方策についても検討を進める。

使用上の注意記載状況・その他参考事項等

解凍赤血球濃厚液「日赤」
照射解凍赤血球濃厚液「日赤」

血液を介するウイルス、
細菌、原虫等の感染
vCJD等の伝播のリスク

Effects of skin disinfection method, deviation bag, and bacterial screening on clinical safety of platelet transfusions in the Netherlands

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BACKGROUND: Bacterial contamination of blood products is a great hazard for development of fatal transfusion reactions. Bacterial screening of platelet concentrates (PC) by aerobic and anaerobic culturing (BacT/ALERT, bioMérieux) was introduced in the Netherlands in October 2001.

STUDY DESIGN AND METHODS: In November 2002, a nationwide, uniform skin cleansing method was introduced with a double-swab disinfection with 70 percent isopropyl alcohol. One location routinely used an integrated diversion bag to collect the first 20 to 30 mL.

RESULTS: Over the calendar years 2002 and 2003, in total 113,093 PCs derived from pooled buffy coats were screened. After introduction of the new disinfection method, 0.85 percent were initially positive. This was a small reduction compared to the previous disinfection methods under which 0.95 percent were initially positive. The location with use of the diversion bag showed a significantly lower frequency of bacterial contamination, with 0.50 percent before and 0.37 percent after introduction of 70 percent isopropyl alcohol. In addition 8000 apheresis PCs were also screened, showing 24 initially positive samples (0.30%).

CONCLUSION: The use of the diversion bag and, to a lesser extent, the use of double swabs with 70 percent isopropyl alcohol, led to a reduction of contamination. As expected, predominant contamination with resident skin bacteria was reduced. The combination of diversion bag and new disinfection led to a frequency of initial positive results for pooled five-donor PCs, which is similar to that of single-donor apheresis PCs. Furthermore, the bacterial detection system and associated product recall procedures have been shown to be effective in preventing transfusion of contaminated PCs and/or related red cells, especially for rapidly growing bacteria.

Since the dramatic reduction of transfusion-transmitted viral infections though screening for various blood-borne viruses, transfusion-related bacterial infections have become one of the major risks of transfusion. Bacterial contamination is considered to be, after clerical errors, the second most common cause of death from transfusion, with mortality rates for platelet (PLT)-related sepsis ranging from 1 in 20,000 to 1 in 100,000 donor exposures.[1-3] In contrast, the current frequencies of virus transmission via blood components are estimated as 1 in 1,800,000 for hepatitis C virus, 1 in 220,000 for hepatitis B virus, and 1 in 2,300,000 for human immunodeficiency virus.[4,5] Therefore, additional screening of blood products for bacterial contamination is under consideration or has already been implemented in many countries, both in Europe and in North America.[6]

Because of their storage at room temperature, PLT products are most sensitive for bacterial contamination and thus a logical choice to start screening. In the Neth-

ABBREVIATIONS: CNS = coagulase-negative staphylococci; IPA = isopropyl alcohol; PC(s) = platelet concentrate(s); T5 = PCs prepared from pools of five buffy coats; TTBI = transfusion-transmitted bacterial infection.

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erlands, nationwide screening for bacterial contamination in 100 percent of PLT products was introduced at the end of 2001. All Dutch blood banks use the BacT/ALERT culturing system (bioMérieux [formerly Organon Teknika], Boxtel, the Netherlands), with a standardized protocol.

It has previously been shown that in the majority of positive cultures, bacterial contamination is a result of resident skin flora[7] most likely originating from the venipuncture plug.[8,9] Although hygienic precautions are taken to prevent contamination during collection, a further reduction of the number of products with bacterial contamination is desirable. To reduce the risk of contamination by skin flora two strategies can be used: 1) diversion of the first aliquot of the donation and 2) improvement of skin disinfection.

Regarding the first method we, as well as others, have previously shown that diversion of the first 10 mL of a whole-blood donation reduces the incidence of bacteria in the remaining whole-blood unit.[8,10,11] The effect of this diversion, however, on bacterial contamination of the final product, PLT concentrates (PCs) prepared from pooled buffy coats, has not yet been reported.

Considering skin disinfection, iodine is the most effective disinfectant. Because it is considered to be a donor-unfriendly agent, however, isopropyl alcohol (IPA) is the next best choice. McDonald and coworkers[12] have shown that improved skin disinfection methods drastically reduced the number of remaining bacteria on the phlebotomy puncture site—especially those methods with a double-swab method, with the best results for the combination of IPA and iodine. For this approach too, the final effect on bacterial contamination of buffy coat–derived PCs has not yet been reported.

From January 2002, at collection centers of the Sanquin Blood Bank Region Southeast (Nijmegen, the Netherlands), a collection system with an integrated diversion bag was used to divert the first 20 to 30 mL of the whole-blood donation. This volume was subsequently used for infection disease and immunohematology testing. All other collection centers of the Sanquin Blood Banks used standard whole-blood collection systems without a diversion bag. In October 2002, a standardized skin disinfection method, with 70 percent IPA in a double-swab method, was introduced in all the collection centers of the Sanquin Blood Banks (including Nijmegen). During the whole period all apheresis PCs were collected with a diversion pouch included in the system.

In this report we evaluate data on bacterial contamination in the Netherlands for all apheresis PCs and PCs prepared from pools of five buffy coats (T5), collected in 2002 and 2003. The large numbers enable us to make a reliable judgment of the effect of diversion and/or changed disinfection method on the final degree of bacterial contamination of PC.

MATERIALS AND METHODS

Blood collection with or without diversion

Whole blood was collected under standard blood banking conditions with either a four-bag top and bottom citrate phosphate dextrose (CPD)-saline adenine glucose mannitol (SAGM) red cell (RBC) inline filter system (Compoflex, Fresenius Hemocare, NPBI International, Emmer-Compascuum, the Netherlands) or a comparable system (Baxter PL146-CPD-70 mL 3-Optipure, Baxter, Utrecht, the Netherlands).

Sanquin Blood Bank Region Southeast (Nijmegen, the Netherlands) used a five-bag top and bottom CPD-SAGM RBC inline filter system (Compoflex, Fresenius Hemocare, NPBI International) including an integrated sample bag (T3941, Fresenius Hemocare), in which diversion of the first 20 to 30 mL of the donation was performed. After collection of the first volume, a clamp was set and the sample bag was sealed. Donation proceeded normally in the collection bag with CPD. All other collection centers collected blood for infectious diseases and immunohematology testing after donation was completed via a sampling site attached to the collection system.

Processing to PCs

Whole blood is processed similarly at all collection centers after collection, with rapid cooling to 20°C and overnight storage at this temperature.[13] Briefly, after a hard centrifugation of the whole blood, the buffy coats are collected. Five buffy coats (same blood group) are pooled and mixed with either 300 mL of plasma or additive solution (AS; PAS I, Baxter). The pooled buffy coats are centrifuged again (soft centrifugation) to produce T5 products. Preparation procedures for T5 varied slightly between regions, but these differences (type of leukodepletion filter and storage container) are not likely to cause variations in the degree of bacterial contamination.

The blood bank locations of Rotterdam (Region Southwest), Utrecht (Region Northwest), and Nijmegen (Region Southeast) used PAS II as AS in the T5 products; all other blood bank locations used plasma for pooling.

Skin disinfection

Before October 2002 skin disinfection was performed with various methods in the Sanquin collection centers, referred to as the "old skin disinfection method." Most centers (>95% of total collections) used a single swab method with 70 percent alcohol–0.5 percent chlorhexidine or 70 percent IPA, but some small centers used single swabs with iodine tincture, whereas one small center used a double-swab method with 70 percent alcohol–0.5 percent chlorhexidine. Because no differences were found between the various centers for the old methods,