別紙様式第2－1

医薬品　研究報告　調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分		総合機構処理欄
一般的名称	－		研究報告の公表状況	http://www.guardian.co.uk/frontpage/story/0,,1765531,00.html		公表国 英国	
販売名(企業名)	－						
研究報告の概要	英国政府は1990年代に輸出した英国製汚染血漿分画製剤により、患者がvCJDを発症するリスクにさらされていると14ヵ国(ブラジル、トルコ、ブルネイ、アラブ首長国連邦、インド、ヨルダン、オマーン、シンガポール、ベルギー、モロッコ、エジプト、フランス、オランダおよびイスラエル)に警告した。 問題は血漿分画製剤が数千人の血液から製造されいていることであり、科学者は未発症の感染者の供血によって引き起こされる「第二波」の災害を懸念している。 血液を介した感染リスクは2003年12月までは仮説に過ぎなかったが、その後輸血を介して感染した英国人患者が出現し、さらに2例が見いだされていることから、保健当局は国立企業のBio Products Laboratory (BPL) により国外に輸出された血液製剤を再調査しなければならなくなった。 保健保護局は、輸出量や危険性を勘案し、最も危険性の高いブラジルとトルコ、それより危険性は低いが予防措置を講じる必要がある6ヵ国(ブルネイ、アラブ首長国連邦、インド、ヨルダン、オマーンおよびシンガポール)へは、予防措置(患者を追跡し、血液や臓器を提供しないよう通知すること、治療を必要とする場合は医師や歯科医に知らせるよう通知すること)を講じるように保健省に勧告した。危険性の低いベルギー、モロッコ、エジプトと、血液製剤を製造できるフランス、オランダおよびイスラエルについては自ら評価するよう勧告した。 BPLを管理するNHS血液・移植当局は「現在のところ血漿分画製剤と関連づけられたvCJD症例はない。」としている。						**使用上の注意記載状況・その他参考事項等** **重要な基本的注意** 現在までに本剤の投与により変異型クロイツフェルト・ヤコブ病(vCJD)等が伝播したとの報告はない。しかしながら、製造工程において異常プリオンを低減し得るとの報告があるものの、理論的なvCJD等の伝播のリスクを完全には排除できないので、投与の際には患者への説明を十分行い、治療上の必要性を十分検討の上投与すること。

報告企業の意見	今後の対応
vCJD多発国である英国が14ヵ国に血漿分画製剤を輸出していたので、該当国に対し警告を発したとの報告である。報告中でNHSは「現在のところ血漿分画製剤と関連づけられたvCJD症例はない。」と述べている。 なお、当社では英国より血漿分画製剤又はその原料血漿を輸入したことはない。	今後ともvCJDに関する安全性情報、規制情報等に留意していく。

NIHONSEIYAKU
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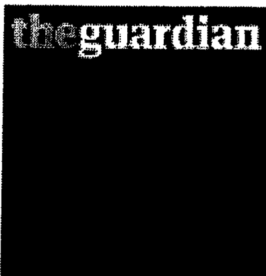

British blood products may pose vCJD risk in 14 countries

- UK issues warning on 'mad cow disease'
- Documents show Brazil and Turkey are high on list

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James Meikle and Rob Evans
Tuesday May 2, 2006
The Guardian

The government has been forced to warn 14 countries that patients are in danger of developing the human form of mad cow disease as a result of contaminated British blood products sold abroad.

Documents released under the Freedom of Information Act show that patients in Brazil and Turkey are most at risk from the products, although it is too early to know how many, if any, foreign patients may develop the incurable variant CJD, as it takes many years to appear. The Turkish authorities said they had traced patients at risk and were closely monitoring them, while Brazil would not comment.

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The contaminated blood products were exported in the 1990s by the British government to treat conditions such as haemophilia, severe burns and immune deficiency. At the time the government considered there was no risk.

Twenty-eight people abroad have already developed vCJD by eating cattle meat from Britain infected with BSE. However, the dangers of another route of transmission are now becoming more evident. Scientists are worried about a "second wave" of casualties caused by blood donated by people infected but not yet displaying symptoms of the disease.

The risk of passing on the disease in this way was considered only theoretical until December 2003, when it emerged that a patient in Britain had been infected through a blood transfusion, leading to new safety measures. Another two cases have since been identified. Health authorities then had to re-examine blood products sent abroad by the state-owned company Bio Products Laboratory (BPL).

The documents show that, following the rethink, the Health Protection Agency was concerned "about the potential infectivity of blood". Believing the potential risk of vCJD to be "very uncertain", the agency advised the Brazilian and Turkish health ministries to take precautions to reduce the possibility of spreading vCJD as "sufficient quantities" of the "at-risk" products had been exported.

These measures included tracking down patients and telling them not to donate blood, organs or tissues. Patients are also told to inform doctors and dentists if they need any treatment.

In Britain, up to 6,000 people were considered to be at risk. The problems stem from the way blood products are made, from processing thousands of separate donations. The concerns arise from just 23 donations made by nine

people who went on to develop vCJD, showing how minute amounts may be infectious.

The NHS Blood and Transplant Authority, which is responsible for BPL, said: "So far no vCJD cases have been linked to plasma products ... The use of products derived from British blood plasma was ended in 1999 as a precautionary safety measure because of what were then regarded as only theoretical risks. But cases where patients might have been put at risk before that date have since come to light as further cases of vCJD have been diagnosed in people who were blood donors. Since 2004, no one who received a blood transfusion after 1980 has been allowed to donate blood themselves."

The Health Protection Agency decided that patients in six countries - Brunei, UAE , India, Jordan, Oman and Singapore - had been put in less jeopardy than those in Brazil and Turkey, but might need to take precautions. Less dangerous batches were imported by Belgium, Morocco and Egypt. France, Holland and Israel were advised to carry out their own assessments, as manufacture of the blood products was completed in their countries. The French government concluded that there was no danger from the products, which were re-exported to 10 unnamed countries.

The Guardian has previously reported that patients worldwide may have been exposed to vCJD, but the documents detail for the first time the countries, the amounts and the risk assessments. British authorities cannot say how many patients abroad may now be in danger.

There have been 161 cases of vCJD in Britain. There are 15 cases in France, four in Ireland, two in the US, and one each in Canada, Italy, Japan, the Netherlands, Portugal, Saudi Arabia and Spain.

Some of these victims are known to have caught vCJD by eating infected beef in Britain. Most others live in countries that have also had outbreaks of BSE that may well have originated from Britain.

Graham Steel, whose brother Richard died from vCJD, drew parallels to the spread of BSE. "[It is] eerily reminiscent of the 1980s when 'theoretically' infectious meat and bonemeal was exported by the UK around Europe and beyond despite the fact that the risks of spreading diseases were known about in 1972-73. A total recall was deemed too expensive."

Special reports

2

別紙様式第2-1

医薬品 研究報告 調査報告書

識別番号・報告回数		1	報告日	第一報入手日 2006年4月10日	新医薬品等の区分		厚生労働省処理欄
一般的名称	別紙のとおり		研究報告の公表状況	Identification of a Novel Single-Stranded DNA Fragment Associated with Human Hepatitis J. Inf,,Dis. 15;193(8):1089-97. 2006		公表国 日本	
販売名(企業名)	別紙のとおり						
研究報告の概要	(問題点:原因不明の急性肝炎発症患者の血液から、A型からE型ではない未知のDNA配列を持つ「NV－F」感染症が確認された。) 原因不明の急性肝炎を発症した患者の血液から、未知のDNA配列「NV-F」を発見。A型からE型までの肝炎ウイルスが検出されない、原因不明の肝炎患者69人中の17人(24.6%)で、NV-Fが検出された。 17人のうち1人は劇症肝炎で、NV-Fは発症から約10日間、血中に現れ、症状の回復につれて消えた。この患者の肝細胞からは、NV-Fが作り出した抗原が検出され、NV-Fが肝臓で増殖したことをうかがわせた。 NV-Fは、B型やC型と同じ経路で感染しやすいウイルスのDNAと推測される。						使用上の注意記載状況・その他参考事項等 記載なし
報告企業の意見			今後の対応				
別紙のとおり			現時点においては、特段の対応は不要と考えるが、今後とも関連情報の収集に努め、本剤の安全性の確保を図っていきたい。				

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INF2006-001

MAJOR ARTICLE

Identification of a Novel Single-Stranded DNA Fragment Associated with Human Hepatitis

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By performing nonspecific polymerase chain reaction followed by elimination of chromosome-derived sequences, foreign DNA fragments were obtained from the serum of a patient with non–A–E hepatitis. One of the sequences, named NV-F, contained a partial open reading frame and was detected in 17 (24.6%) of 69 patients with non–A–E hepatitis, including 1 with fulminant hepatitis (vs. in 5 [2.8%] of 180 healthy individuals). A peptide was synthesized accordingly, to detect serum anti–NV-F antibody, which was found in 49 (75.4%) of 65 patients positive for NV-F. This DNA fragment was sensitive to S1 nuclease digestion. Cesium chloride gradient analysis revealed that the NV-F–associated particles had buoyant densities of 1.33–1.39 and 1.22–1.25 g/mL. Immunofluorescence analysis revealed that the novel antigen was present in the hepatocytes of patients infected with NV-F. In conclusion, we have identified a novel single-stranded DNA fragment derived from a virus-like agent associated with human hepatitis.

Previously, when diagnostic tests for the detection of hepatitis A and B viruses (HAV and HBV) were globally available, it had been recognized that a significant proportion of patients with acute and chronic hepatitis were not infected with either virus, and the diseases were referred to as "non-A, non-B hepatitis" [1]. Owing to technological advances in molecular biology, hepatitis C and hepatitis E viruses (HCV and HEV) were subsequently discovered to be the major causes of parenteral and enteric non-A, non-B hepatitis, respectively [2]. Despite this significant progress, the etiology of acute and chronic hepatitis in a substantial number of patients remains unknown. In our previous studies, we found that 15.9% of hospital inpatients with acute hepatitis had non–A–E hepatitis [3]. Additionally, 9.7% of patients with fulminant hepatitis had non–A–E hepatitis [4]. Another study indicated that no definite etiology could be found in 4.9% of patients with chronic hepatitis or cirrhosis; these cases were termed "cryptogenic" [5]. Approximately half of these patients had received transfusions, which supported a virological etiology. Furthermore, enhanced HLA expression in liver samples from patients with chronic non–A–C hepatitis has been reported, which also supports a virological etiology [6]. Therapeutic trials using interferon-α to treat chronic non–A–C hepatitis have consistently resulted in an ~50% response rate, indicating a viral pathogen [7]. Inspired by these observations, scientists struggled to unearth the theoretically existing hepatitis viruses. As a result, several new viruses, including GB virus type C (GBV-C) [8], TTV [9], and SEN virus [10], were discovered. However, epidemiological data failed to confirm a causative role for these viruses in hepatitis. In addition, a high percentage of individuals infected by these viruses were found to be healthy carriers. Furthermore, in some studies, it was argued that GBV-C was not, in fact, a hepatotropic virus [8].

In the present article, we describe a novel agent associated with human hepatitis. Epidemiological data suggest that it is highly associated with non–A–E hepatitis. Biochemical evidence indicates that it is hepatotropic. Additionally, it was detected in a patient with fulminant non–A–E hepatitis.

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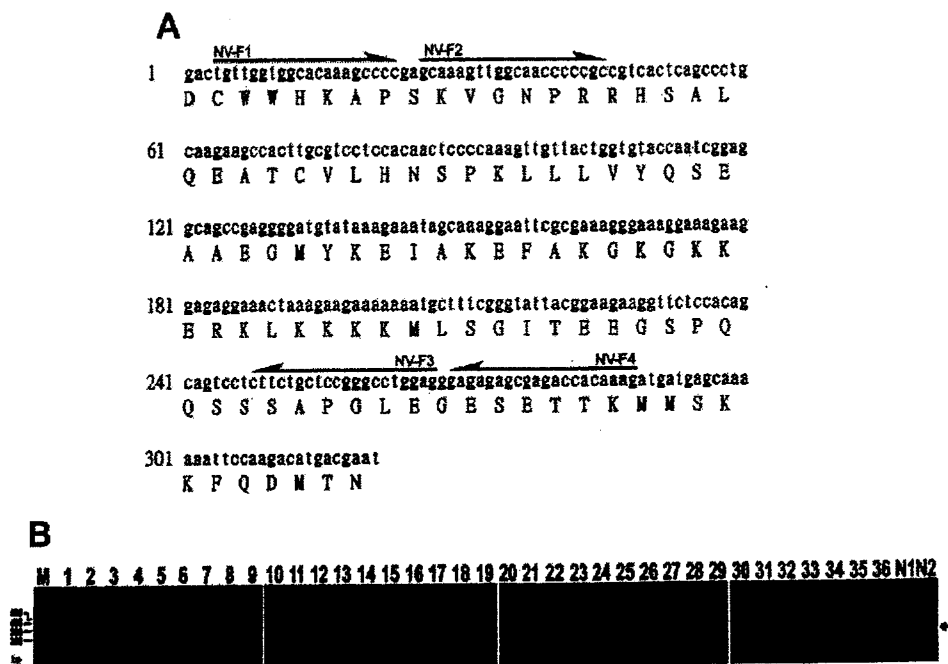

Figure 1. Identification of a foreign sequence in patients with non–A–E hepatitis. *A*, Nucleotide sequence of the NV-F DNA fragment and conceptual translation of the putative partial reading frame. The positions of 4 primers (NV-F1 to NV-F4) used for polymerase chain reaction (PCR) detection of NV-F 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

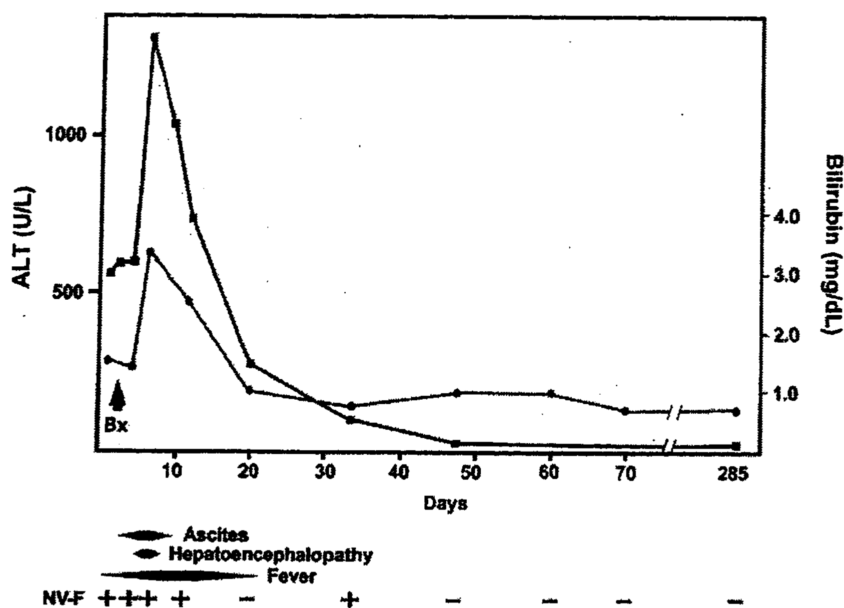

Figure 2. Clinical course in a patient with fulminant non–A–E hepatitis. Squares denote alanine aminotansferase (ALT) levels (U/L), and circles denote bilirubin levels (mg/dL). The periods of clinical symptoms are marked with solid bars, and "Bx" indicates the time of the liver biopsy. The NV-F sequence 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

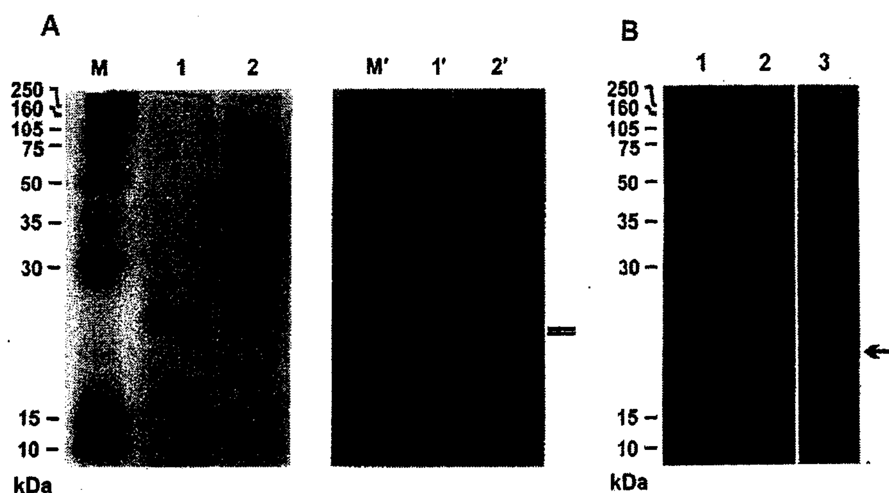

Figure 3. Generation of NV-F peptide and development of antibody against NV-F antigen. *A,* A fusion protein containing polyhistidine, Xpress epitope, and a peptide encoded by NV-F was expressed in insect cells. The protein extract was purified by affinity column and was analyzed by electrophoresis. 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.

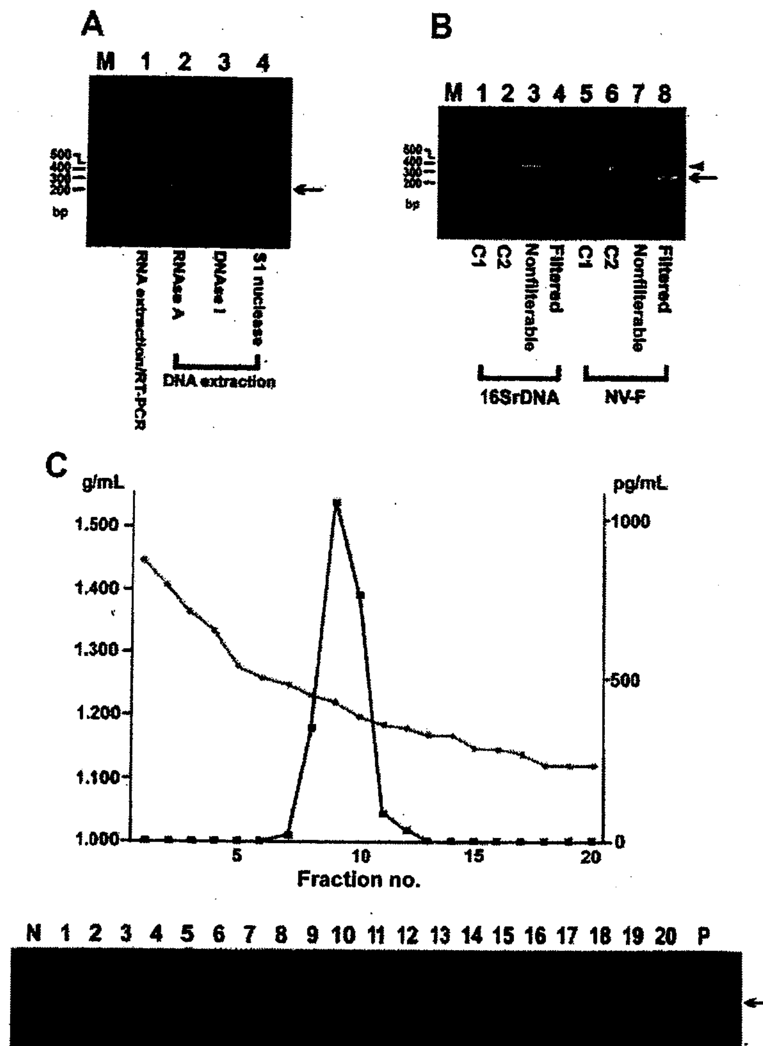

Figure 4. Characterization of the NV-F agent. *A,* Extraction of nucleic acid from the serum sample by either the RNA extraction *(lane 1)* or the DNA extraction method *(lanes 2–4).* After RNA extraction, reverse transcription (RT) polymerase chain reaction (PCR) for detection of the NV-F sequence was 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

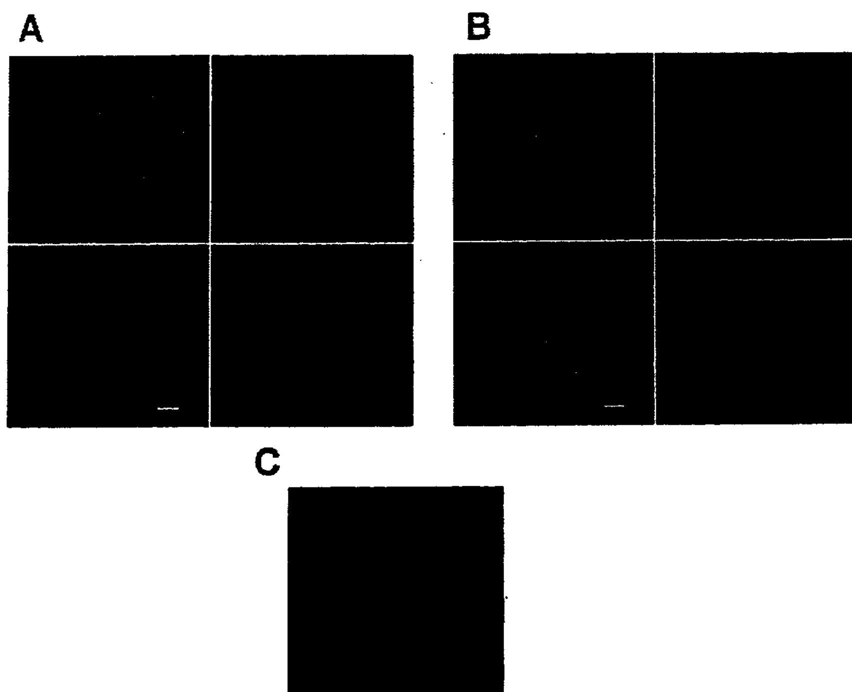

Figure 5. Detection of NV-F antigen in the liver biopsy sample from a patient with fulminant non–A–E hepatitis (patient F). Two different sections (*A* and *B*) from the same biopsy are shown. Positive cells in panel B are shown at higher magnification in panel C. Immunofluorescence analysis was performed 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*

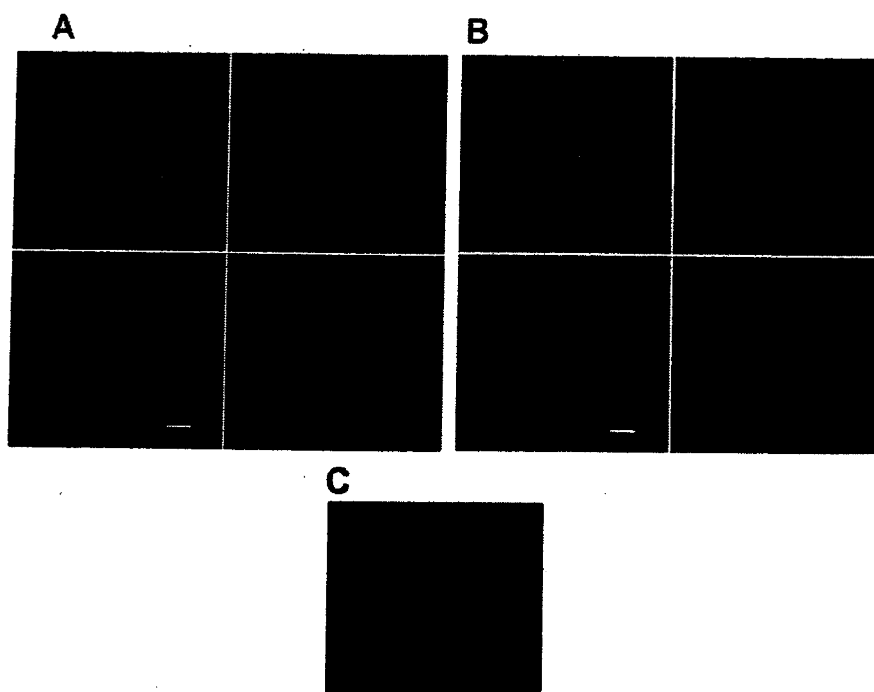

Figure 6. Detection of NV-F antigen in the liver biopsy sample from a patient with NV-F and hepatitis B virus coinfection (patient B). See the legend to figure 5 for further details.

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.

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