level and 3% of individuals with an ALT level of ≥201 IU/l have ongoing subclinical infection of various HEV strains, suggesting the frequent occurrence of subclinical HEV infection, although clinical HEV infection is rarely reported. A large study of individuals who do not have an elevated ALT level is needed to assess the exact frequency of subclinical HEV infection, taking into consideration the geographic region in Japan.

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研究報告の概要	使用上の注意記載状況・その他参考事項等
GBV-C及びHGVは、非B非C型肝炎の原因として関与する新たなウイルスとして紹介されたが、これらウイルス粒子の形態は依然不明であるとともに、非A-E肝炎の殆どの症例は、それらの感染と関係がない。我々は、ALTが高く、HCV抗体とB型肝炎表面抗原が陰性であるドナーからの血漿サンプル中のウイルス様粒子を視覚的に捉えようと試み、このウイルス様粒子と非経口的に感染するGBV-C/HGVの遺伝子との関係を調査した。 HBV及びHCVどちらにも感染していない高ALTレベルの血漿13検体及び正常ALTレベルの血漿10検体の計23検体を40-60%ショ糖密度勾配遠心分離法によって分離し、ウイルス様粒子(VLPs)を電子顕微鏡で観察した。血漿中のGBV-C/HGV RNAを検査した。ウイルス様粒子は、高ALTレベルの血漿13検体のうち12検体（92.3%）及び正常対照血漿10検体のうち1検体（10%）の1.15-1.16g/mLの密度のフラクションから見つかった。VLPsの超微細構造形態は、大きさ、外観とも多形性であった（大部分のVLPsは35-45nmの内核と表面に長さ9-12nmのスパイク様突起を有する50-80nmの球状粒子）。直径50-70nm、長さ110-160nmの棒状のVLPsも同じ検体で観察された。循環血液中のVLPsの検出率は、有意に高ALTレベルと関係（P<0.001）していたが、VLPsを含む血漿のいずれにも、GBV-C/HGV RNAは検出されなかった。HBV及びHCVともに陰性の血漿中に高ALTとの有意な関係が認められた球状のVLPsが確認され、それらが非B非C型肝炎に関係していることが示唆された。	2. 重要な基本的注意 (1) 本剤の原材料となる献血者の血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体、抗HTLV-Ⅰ抗体陰性で、かつALT(GPT)値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査(NAT)を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した血漿を原料として、Cohnの低温エタノール分画で得た画分から人ハプトグロビンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において60℃、10時間の液状加熱処理及び濾過膜処理（ナノフィルトレーション）を施しているが、投与に際しては、次の点に十分注意すること。

報告企業の意見	今後の対応
非B非C型肝炎との関連が示唆されるウイルス様粒子を発見したとの報告である。 非B非C型肝炎との関連が示唆されるウイルス様粒子は、GBV-C/HGVと関連していないこと以外の性状等に関する情報は不足しているため、現時点において安全性評価は困難であり、今後も情報収集に努める。	ウイルス様粒子に関する追加情報の入手に努める。

ハプトグロビン

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ORIGINAL PAPER

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Unidentified virus-like particles are detected in plasmas with elevated ALT levels: are they significant of etiological agent(s) of non-B, non-C hepatitis?

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Abstract GB virus C (GBV-C) and hepatitis G virus (HGV) have been proposed as new viruses etiologically implicated in non-B, non-C hepatitis, but the morphology of these particular virus particles is still unknown, and most cases of non-A to E hepatitis do not relate to their infections. We tried to visualize virus-like particles (VLPs) in plasma samples from hepatitis B surface antigen- and antibody to hepatitis C virus (HCV)-negative blood donors with elevated alanine aminotransferase (ALT), and examined the association of the virus-like particles and the genomes of parenterally transmissible GBV-C/HGV. Twenty-three plasma samples, 13 with elevated ALT levels and 10 with normal ALT values, from blood donors without infections of hepatitis B virus (HBV) and HCV, were subjected to a 20%–60% sucrose density gradient centrifugation, and virus-like particles were observed by electron microscopy. GBV-C/HGV RNAs in the plasmas were tested. Virus-like particles were found in the fractions with densities of 1.15–1.16 g/ml from 12 of 13 (92.3%) plasmas with elevated ALT levels and 1 of 10 (10%) normal controls. The ultrastructural morphology of visualized VLPs was pleomorphic in size and appearance; the majority of the VLPs were 50- to 80-nm spherical particles with a 35- to 45-nm inner core and 9- to 12-nm-long surface spikelike projections. Rodlike VLPs 50–70 nm in diameter with a length of 110–160 nm were also observed in the same samples. The incidence of detection of the circulating VLPs was significantly ($P < 0.001$) related to elevated ALT levels, but GBV-C/HGV RNAs were detected in none of the plasmas containing the virus-like particles. Spherical VLPs are detected in HBV- and HCV-negative plasmas significantly correlated with the elevation of ALT, suggesting that they are implicated in non-B, non-C hepatitis.

Key words Non-B, non-C hepatitis · Virus-like particle · Hepatitis C virus · GB virus C · Electron microscopy

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Introduction

The genomes of hepatitis C virus (HCV), GB virus C (GBV-C), and hepatitis G virus (HGV) have been successfully cloned without isolation of the virus particles.[1-3] The causative role of HCV in bloodborne acute and chronic hepatitis has been well established. Although GBV-C/HGV can be transmitted parenterally, most of their infections are not associated with acute and chronic non-B, non-C hepatitis, and there is some doubt whether GBV-C/HGV replicates in the liver and causes hepatitis.[4,5] During immunogold electron microscopy[6-9] of HBV and HCV particles, we have noticed that some plasma samples from blood donors with elevated alanine aminotransferase (ALT) levels contained virus-like particles (VLPs) that did not react positively with antibodies specific to the HCV envelope protein. The VLPs, thus immunologically distinguished from HCV virion, were also different in morphology from the latter, and their etiological implications were not clear. In this article, we have tried to visualize VLPs in hepatitis B surface antigen (HBsAg)- and anti-HCV-negative plasmas and evaluated whether the circulating VLPs were detected with or without a relationship to elevated plasma ALT levels or to GBV-C/HGV RNAs.

Materials and methods

Twenty-three blood donor plasma samples were the subjects in this study. Clinical characteristics of the blood donors are summarized in Table 1. These samples were negative for HBsAg (AUSRIA II-125; Dainabot, Tokyo,

Table 1. Summarized data of detection of virus-like particles and nucleic acids of known hepatitis viruses in plasma samples negative for HBsAg and anti-HCV

Plasma no.	ALT (IU/l)	Virus-like particles[a]		HBV DNA[b]	HCV RNA[c]	GBV-C RNA[d]	HGV RNA[e]
		Spherical	Rod-like				
1	91	–	–	–	–	–	–
2	81	2+	–	–	–	ND	ND
3	87	2+	–	–	–	–	–
4	70	3+	2+	–	–	–	–
5	82	3+	2+	–	–	–	–
6	103	3+	1+	–	–	–	–
7	121	3+	3+	–	–	–	–
8	67	2+	1+	–	–	–	–
9	117	2+	–	–	–	–	–
10	72	2+	1+	–	–	–	–
11	103	2+	–	–	–	–	–
12	78	3+	1+	–	–	ND	ND
13	77	1+	–	–	–	–	–
14	4	1+	–	–	–	–	–
15	28	–	–	–	–	–	–
16	6	–	–	–	–	–	–
17	10	–	–	–	–	–	–
18	7	–	–	–	–	–	–
19	11	–	–	–	–	–	–
20	4	–	–	–	–	–	–
21	6	–	–	–	–	–	–
22	6	–	–	–	–	–	–
23	8	–	–	–	–	–	–

ALT, alanine aminotransferase (normal < 35 IU/L); ND; not dated
[a] Circulating virus-like particles (VLPs) in a 5-square section of a 300-mesh grid was photographed under electron microscopy. The mean numbers of VLPs in one square was indicated as – (negative), 1+ (0 < + < 10), 2+ (10 ≦ 2+ < 100), and 3+ (100 ≦ 3+)
[b] HBV DNA was tested using a Quantiplex HBV-DNA assay kit
[c] HCV RNA was tested using an Amplicor HCV kit
[d] GBV-C and [e] HGV RNAs were assayed as described in the text

Japan), antibody to HBV core antigen (CORAB; Dainabot), HBV DNA (Quantiplex HBV-DNA Assay; Chiron, Emeryville, CA, USA), anti-HCV (Ortho HCV Ab IRMA Test III; Ortho-Clinical Diagnostics, Tokyo, Japan), HCV RNA (Amplicor HCV; Roche Molecular Systems, Branchburg, NJ, USA), and antibodies to human T-cell leukemia virus type I (Determiner HTLV-I Antibody; Kyowa Medex, Tokyo, Japan) and human immunodeficiency virus (GENELAVIA MIXT; Sanofi Diagnostics Pasteur, Marnes la Coquette, France). Thirteen samples were elevated in alanine aminotransferase (ALT) levels (≧35 IU/l), and 10 samples were within a normal level in ALT (<35 IU/l). Total cholesterol and triglyceride levels were normal in all plasma samples.

One hundred milliliters of each plasma sample was diluted with two volumes of TEN [100 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl] and centrifuged at 75 000 *g* for 6 h at 4°C. A suspension of the pellet in TEN was centrifuged again at 150 000 *g* for 2.5 h at 4°C. An approximately 1000-fold-concentrated suspension of the sample in TEN was layered on a 20%–60% (W/W) linear sucrose density gradient in TE (100 mM Tris-HCl, pH 8.0, 1 mM EDTA) and centrifuged at 100 000 *g* for 16 h at 4°C, which was followed by fractionation from the bottom of the tube. After measurement of the sucrose concentration of every fraction, each sucrose fraction was diluted in phosphate-buffered saline (PBS, pH 7.4) and then centrifuged at 150 000 g for 2.5 h at 4°C. The resulting pellet was suspended in 100 ml PBS, equivalent to 1:1000 of the original plasma volume, for observing VLPs, and stored at –80°C until use. Two to three microliters of the concentrated specimen was mounted on a Formvar-coated and carbon-vaporized copper grid and examined under a Hitachi H-800 electron microscope operated at 100 kV after staining with 2% phosphotungstic acid (pH 6.5). To observe the nucleic acid-containing virus core structure, the specimen was subjected to repeated freezing and thawing several times, and then stained with 0.2% uranyl acetate (pH 4.4).

The GBV-C genome was assayed by reverse transcription-nested polymerase chain reaction (PCR) using primers derived from the NS3/helicase region of GBV-C genome reported by Simons et al.,[2] and this assay was performed as described elsewhere.[10] The genome of HGV, provisionally designated by Linnen et al.,[3] was assayed by using the kit of Boehringer Mannheim (Mannheim, Germany), which contained one primer pair for the NS5A region and a second primer pair for the 5′-untranslated region as well as the corresponding capture probes, according to the manufacturer's instructions.

Fisher's exact probability test was used to assess the significance of differences between the sample groups with or without elevated ALT levels.

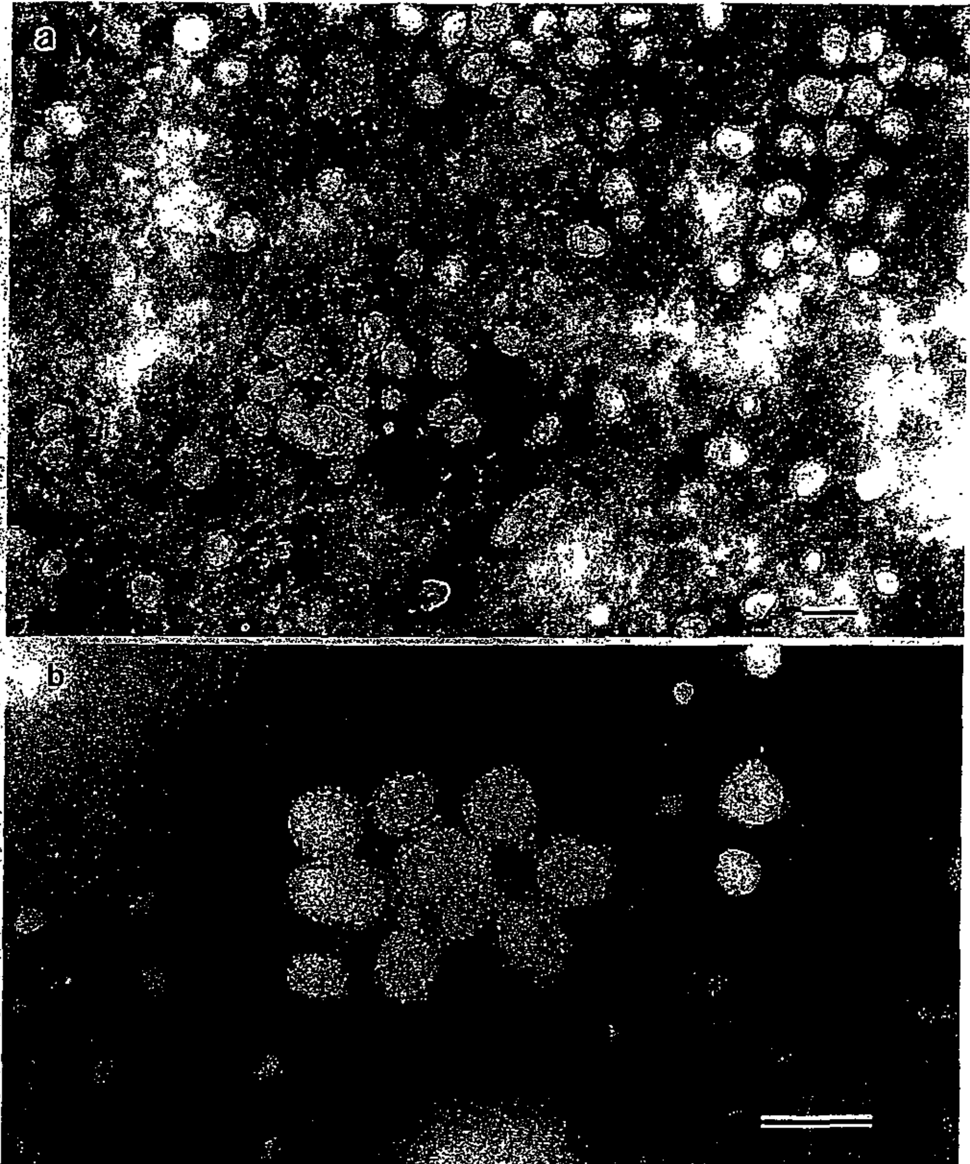

Fig. 1. Electron micrograph of negatively stained 50- to 80-nm spherical virus-like particles (VLPs) in hepatitis B surface antigen (HBsAg)- and anti-hepatitis C virus (HCV)-negative plasma with an elevated alanine aminotransferase (ALT) level. VLPs from sample no. 6 (a) and sample no. 3 (b) are shown. *Bar* 100 nm

Results

Spherical VLPs were detected in 12 of 13 (92.3%) plasma samples with elevated ALT levels and 1 of 10 (10%) plasma samples with normal ALT values. The incidence of detection of the virus-like particles was significantly ($P < 0.001$) related to the elevation of plasma ALT levels (see Table 1).

Most the visualized VLPs were spherical particles 50–80 nm in diameter with 9- to 12-nm-long surface spikelike projections (Fig. 1a,b). Some VLPs have a large diameter, more than 100 nm (Fig. 1a). In some specimens were detected more than a moderate number of VLPs, and rodlike VLPs 50–60 nm in diameter with a length of 110–160 nm were also detected that had surface projections similar to those of the 50- to 80-nm spherical particles (Fig. 2). The diameter of the rodlike VLPs was 50–70 nm and their length was 110–160 nm. In addition, a 35- to 45-nm corelike structure containing the electron-dense material within a spherical VLP (Fig. 3a,b) and two inner cores of a rodlike VLP (Fig. 3c) were visualized by positive staining with uranyl acetate. These VLPs were found to be constantly banded in a sucrose density gradient at around 1.15–1.16 g/ml (range, 1.12–1.18 g/ml). GBV-C/HGV RNAs were not detected in any of the plasma samples (see Table 1).

Discussion

We found VLPs in ALT-elevated plasma samples from blood donors without infections of HBV and HCV that were consistently banded at around 1.15–1.16 g/ml by

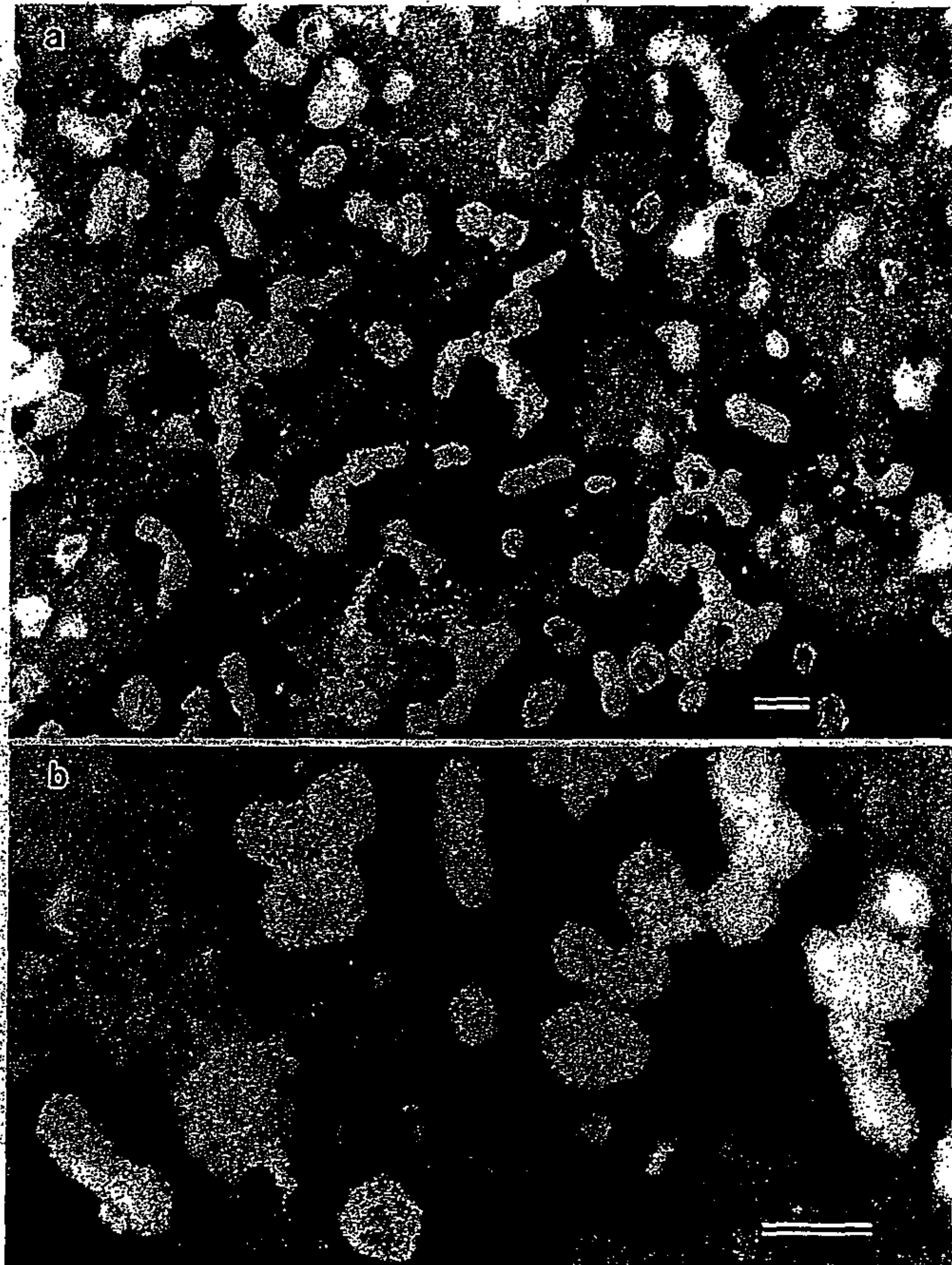

Fig. 2. Negatively stained electron micrograph of rodlike VLPs presented in plasma containing a moderate number of the spherical VLPs. Rodlike VLPs from sample no. 7 (a) and sample no. 5 (b) are shown. *Bar* 100 nm

sucrose density gradient centrifugation. The majority were 50- to 80-nm spherical particles with 9- to 12-nm-long surface projections (see Fig. 1), and were, if anything, morphologically resembling togaviruses or coronaviruses. Positive staining revealed that the electron-dense material combined with uranium existed in a 35- to 45-nm internal core structure of the spherical virus-like particle, and indicated that the spherical particles had the nucleic acid therein (see Fig. 3). In addition, rodlike VLPs (Fig. 2) were observed concomitantly in the specimens containing many spherical VLPs, and the surface spikelike projections of rodlike forms looked similar to those of the spherical particles. Interestingly, two internal cores (Fig. 3c) were detected in a rodlike form, suggesting that this form could be a diploid particle of the virus. Thus, we might consider that both the spherical and the rodlike virus-like particles belong to the same virus species.

In the past three decades, togavirus-like particles have been detected in acute-phase serum from a hemodialysed patient with non-A, non-B hepatitis and in the acute-phase urine of two icteric non-A, non-B hepatitis cases,[11] or in the liver of a patient with sporadic non-A, non-B fulminant hepatitis,[12] but the number of those objects was too small, and thus the etiological implications were not developed. Circulating VLPs, as presented here, were highly prevalent (92.3%) in ALT-elevated plasma samples, and were relatively easy to visualize by conventional electron microscopy, whereas in only one of ten normal controls were a few