

GB virus C E2 glycoprotein: expression in CHO cells, purification and characterization

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A 315 amino acid recombinant segment of the GB virus C (GBV-C) E2 envelope glycoprotein (E2-315) was expressed and secreted from CHO cells. E2-315 was purified by affinity chromatography using a monoclonal antibody directed to a FLAG sequence genetically engineered onto the C terminus of the recombinant protein. The secreted protein had a molecular mass of 48–56 kDa and was shown to be N-glycosylated. Amino acid sequencing confirmed the expected N-terminal sequence. Purified E2-315

was used to develop an ELISA for detection of E2 antibodies in human sera. Antibodies to GBV-C E2 appeared to be directed toward conformational epitopes since human sera reactivity was detected in ELISA using native E2-315, but it was extremely weak or non-existent with denatured E2 protein. The use of an ELISA which can detect human GBV-C E2 antibodies will be important in further understanding of the clinical significance and epidemiology of GBV-C.

Introduction

Approximately 10–20% of parenterally and community acquired hepatitis cases are of unknown cause. Clinical studies suggest that some of these cases may be of viral origin (Alter *et al.*, 1992; Tassopoulos *et al.*, 1992; Fagan *et al.*, 1992; Peters *et al.*, 1993; Thiers *et al.*, 1993; Buti *et al.*, 1994; Kuwanda *et al.*, 1994; Sallie *et al.*, 1994). GB virus C (GBV-C) is one of three newly described flavi-like viruses that is a potential aetiological agent for viral hepatitis (Simons *et al.*, 1995 *a, b*; Muerhoff *et al.*, 1995; Leary *et al.*, 1996). Its genome size and organization are similar to that of the other two new flavi-like viruses, GBV-A and GBV-B, and also to hepatitis C virus (HCV) (Simons *et al.*, 1995 *b*; Leary *et al.*, 1996). The recently described hepatitis G virus (Linnen *et al.*, 1996) appears to be an isolate of GBV-C (Zuckerman, 1996). GBV-C has been identified in human sera by RT-PCR (Simons *et al.*, 1995 *b*; Yoshida *et al.*, 1995; Linnen *et al.*, 1996; Dawson *et al.*, 1996). Using RT-PCR, GBV-C has been identified as a parenterally transmitted virus and has been found in individuals diagnosed with non-A–E hepatitis (Simons *et al.*, 1995 *b*; Yoshida *et al.*, 1995; Linnen *et al.*, 1996; Dawson *et al.*, 1996). It frequently establishes a persistent viraemia in

infected individuals (Dawson *et al.*, 1996; Masuko *et al.*, 1996; Alter *et al.*, 1997 *a, b*) and has also been found in serum of injection drug users (Linnen *et al.*, 1996; Dawson *et al.*, 1996) and transfusion recipients (Linnen *et al.*, 1996; Dawson *et al.*, 1996).

Data from previous studies indicated that approximately 25% of GBV-C RNA positive individuals produced antibodies reactive with prokaryotically expressed recombinant GBV-C proteins (Pilot-Matias *et al.*, 1996 *a*; Dawson *et al.*, 1996). However, epitopes dependent upon protein folding or upon post-translational modifications such as glycosylation would have been absent in these recombinant proteins. Therefore, it was reasoned that mammalian cell expression of GBV-C E2 should be attempted using the same vector and Chinese hamster ovary (CHO) cell line as used successfully for HCV E2 (Lesniewski *et al.*, 1995).

Here we describe expression of a recombinant GBV-C E2 protein, referred to as E2-315, which was truncated at the C terminus to remove a hydrophobic putative membrane-anchoring domain in a strategy analogous to that used successfully to obtain secretion of recombinant HCV E2 protein (Spaete *et al.*, 1992; Lesniewski *et al.*, 1995). The E2-315 protein was secreted and shown to be N-glycosylated. It was also purified and characterized. Previously, we have described that E2-315 and another similar GBV-C E2 recombinant

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protein (E2-336), expressed transiently in a different cell line, are markers of GBV-C clearance from human sera (Pilot-Matias *et al.*, 1996*b*; Dille *et al.*, 1997). Data presented here indicate that the native recombinant GBV-C E2 protein, whether secreted or intracellular, is far superior to denatured GBV-C E2 in its ability to detect GBV-C E2 antibodies in human sera.

Methods

■ Animal and human sera and antibodies. Generation and specificity of rabbit GBV-C E2 antiserum 10363 (directed against a CKS fusion of the E2 region comprising amino acids 209–420 of the GBV-C E2 polyprotein) and rabbit GBV-C E2 antiserum 10365 (directed against a synthetic 30-mer peptide of the E2 region comprising amino acids 335–364 of the GBV-C polyprotein) are described elsewhere (Pilot-Matias *et al.*, 1996*b*). Anti-FLAG M2 monoclonal antibody was obtained from Eastman Kodak. Human sera samples obtained from volunteer blood donor populations, populations considered at risk of acquiring GBV-C and non-A–E hepatitis patients, were as described previously (Dawson *et al.*, 1996). The volunteer blood donor samples were screened and found to be negative for hepatitis B surface antigen and negative for antibodies to hepatitis B core and to HCV as tested by Abbott Laboratories commercial tests. Furthermore, serum alanine aminotransferase (ALT) levels were considered acceptable in blood donation (< 50 IU/L). Biotinylated goat anti-rabbit (H+L), anti-mouse (H+L) and anti-human (H+L) IgGs, streptavidin–horseradish peroxidase and horseradish peroxidase-labelled goat anti-rabbit (H+L) IgG were from Kirkegaard and Perry Laboratories. Fluorescein-labelled goat anti-mouse, anti-human and anti-rabbit antibodies were from Jackson Immuno-Research.

■ Recombinant plasmid containing GBV-C E2-315 sequence. The vector used for CHO cell expression has been described previously (Lesniewski *et al.*, 1995). The GBV-C E2 sequence cloned into the vector encoded a 315 amino acid truncated E2 protein representing amino acids 204–518 of the GBV-C polyprotein of the West African GBV-C isolate sequence that is assigned GenBank accession number U36380 (Leary *et al.*, 1996). A sequence (FLAG) encoding the eight amino acids DYKDDDDK (Eastman Kodak) was incorporated at the C terminus of E2. A schematic diagram of the plasmid construct is shown in Fig. 1. DNA sequence confirmation of inserted sequences was performed using the Sequenase version 2.0 kit (US Biochemical). A negative control plasmid comprised the same vector with an insert encoding an unrelated protein.

■ Expression of E2-315 protein. Reagents for cell culture were from Gibco-BRL, unless stated otherwise. Dihydrofolate reductase-deficient (DHFR⁻) CHO cells, DXB-11, were obtained from L. Chasin at Columbia University. The expression plasmid was transfected into the DHFR⁻ CHO cells using the cationic liposome-mediated procedure (Felgner *et al.*, 1987). Cells, cultured in Ham's F-12 medium supplemented with 10% (v/v) foetal calf serum and L-glutamine (1 mM), were freshly seeded at a density of 5–8 × 10⁵ cells per 25 cm² flask and grown to 60–80% confluency for transfection. Twenty µg of DNA was used for transfection, in which Opti-MEM I–Lipofectin–plasmid DNA solution was overlaid onto Opti-MEM I washed cells. The cells were incubated for 2–3 h at 37 °C, after which the Opti-MEM I–Lipofectin–DNA solution was replaced with culture medium for an additional 24 h; transfectants were then selected with Ham's F-12 containing 1 mM L-glutamine but no hypoxanthine, thymidine or glycine (JRH Biosciences) and 300 µg/ml G418 (Gibco-BRL) (selection medium). Amplification of GBV-C E2 and

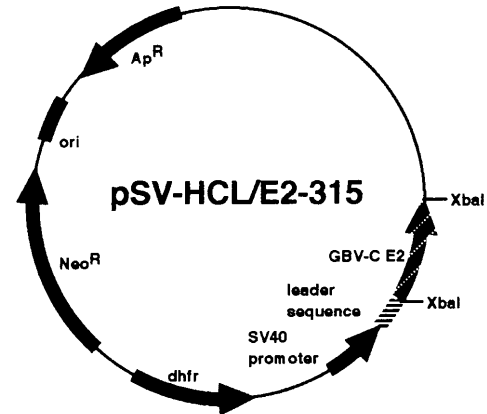


Fig. 1. Schematic of pSV-HCL vector with GBV-C E2-315 insert. The GBV-C sequence was inserted into the unique *Xba*I site of the vector. Ap^R, ampicillin resistance gene; Neo^R, neomycin resistance gene for selection; dhfr, dihydrofolate reductase gene for selection and amplification.

negative control plasmid sequences in DHFR⁺ G418⁺ cells was achieved by stepwise selection with methotrexate (MTX) (reviewed by Schimke, 1988). Cells were incubated in selection medium containing 150 nM MTX (Sigma) for approximately 2 weeks until resistant colonies appeared, and then further amplification was achieved with 5 µM MTX over several weeks. Five µM MTX-adapted cells were used for E2 production. Cultures were expanded into roller bottles. GBV-C E2 antigen production was in protein-free VAS custom medium containing L-glutamine and HEPES without phenol red (JRH Biosciences). Monolayers of CHO cells were overlaid with VAS for several days and then medium was harvested.

■ Immunofluorescence assay (IFA). Five µM MTX-adapted CHO cells were grown on coverslips to approximately 80% confluency in selection medium containing 5 µM MTX. Cells were fixed in 3.7% (v/v) formaldehyde in PBS for 10 min, washed with PBS and then washed several times with IFA buffer (PBS containing 5%, v/v, normal goat serum, 0.1%, w/v, saponin and 0.1%, w/v, sodium azide). Fixed cells were treated overnight at room temperature with primary antibody diluted in IFA buffer. For human sera, dilutions of 1:40 or 1:80 were used and for rabbit 10363 antiserum a dilution of 1:100 was used. Cells were washed again and then reacted with fluorescein-labelled goat anti-primary antibodies at a concentration of 25–40 µg/ml in IFA buffer for 1–2 h at room temperature. After thorough washing, coverslips were mounted inverted on slides for viewing.

■ Purification of E2-315 protein. VAS medium from harvests was clarified by centrifugation at 1500 g for 15 min and filtration through a 0.45 µm cellulose acetate membrane (Nalge), followed by 50–100 × concentration by ultrafiltration using Amicon YM10 membrane. This was followed by exchange into column buffer, 50 mM Tris, 150 mM NaCl, pH 7.5, using a Sephadex G-25 (Pharmacia) column or by dialysis using a Spectra/Por 6000–8000 kDa molecular mass cut-off membrane (Spectrum Medical Industries). The resultant material was loaded onto a FLAG M2 monoclonal antibody affinity column (Eastman Kodak). Non-binding protein was eluted by washing the column with column buffer and bound protein was eluted using an excess of FLAG peptide (in column buffer). A 0.1 M glycine–HCl, pH 3.5 wash of the column was done to elute any protein which remained bound to the column during FLAG peptide elution. Eluted E2-315 was concentrated using Amicon YM10 membranes and free FLAG peptide was removed by gel filtration using

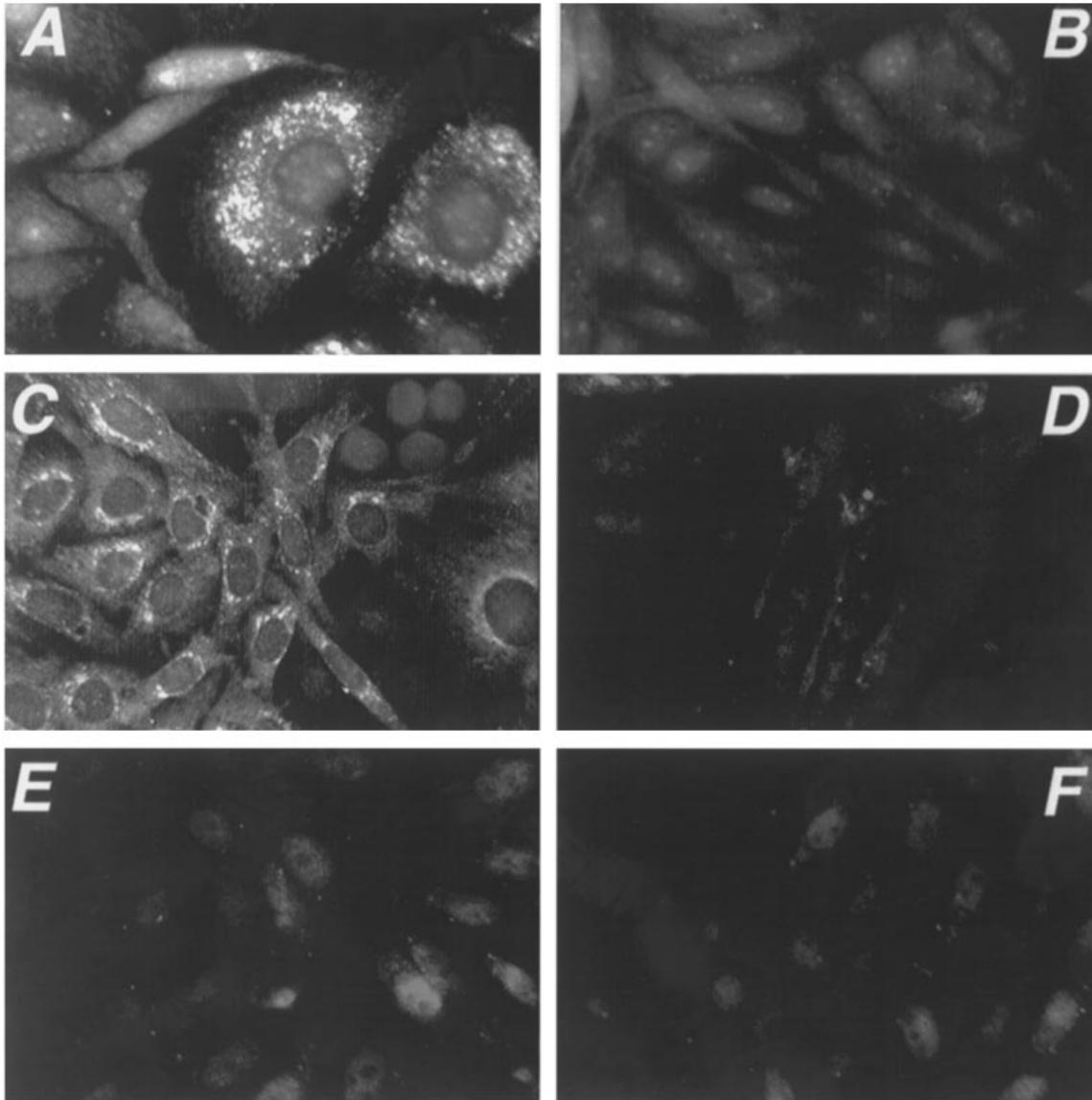


Fig. 2. Immunofluorescence assay of GBV-C E2-315 expression in transfected CHO cells. (A) GBV-C E2-315 plasmid-transfected CHO cells analysed using rabbit antiserum 10363; (B) negative control plasmid-transfected CHO cells analysed using antiserum 10363; (C) GBV-C E2-315 plasmid-transfected CHO cells analysed using human serum sample 20; (D) negative control plasmid-transfected CHO cells analysed using human serum sample 20; (E) GBV-C E2-315 plasmid-transfected CHO cells analysed using human serum sample 25; (F) negative control plasmid-transfected CHO cells analysed using human serum sample 25. Magnification was 400 \times .

Sephadex G-25. Protein concentration used for solid phase immunoassay of human sera was based on estimated absorbance of the purified E2-315 at 280 nm.

■ **SDS-PAGE, Western blotting and dot blotting.** Protein samples were electrophoresed under reducing conditions using 12% SDS-PAGE gels (Laemmli *et al.*, 1970). Gels were either stained with Coomassie Brilliant Blue R-250 or were electrophoretically transferred to ProBlott membrane (Applied Biosystems) (Towbin *et al.*, 1979). For dot blots, native protein or protein which had been denatured by boiling in 3% (w/v) SDS, 0.3 M 2-mercaptoethanol was applied under vacuum to ProBlott membrane using a dot blot apparatus (BRL Life Technologies).

Blots were reacted with 1:300 dilutions of rabbit 10363 or 10365 antiserum, with FLAG M2 monoclonal antibody at 10 μ g/ml or with human sera diluted 1:100. Detection was with biotinylated goat anti-rabbit, anti-mouse or anti-human affinity purified IgG (H+L), respectively, and a streptavidin-horseradish peroxidase/4-chloro-1-naphthol (Sigma) detection system.

■ **Deglycosylation of E2-315.** Both native and denatured E2-315 (boiled in the presence of 0.5% w/v SDS, 50 mM 2-mercaptoethanol and 50 mM EDTA) were treated with N-Glycanase (Genzyme Corp.). Deglycosylation was carried out for 61 h at 37 $^{\circ}$ C. For the denatured sample, the deglycosylation buffer contained 50 mM Tris-HCl, pH 7.5,

0.17% (w/v) SDS, 16.7 mM 2-mercaptoethanol, 16.7 mM EDTA, 1.25% (v/v) NP40 and 0.3 units of N-Glycanase/ μ g protein. For the native sample, SDS and 2-mercaptoethanol were eliminated and 1.2 units of N-Glycanase/ μ g protein were used. Control reactions were performed in the absence of N-Glycanase.

■ **N-terminal amino acid sequencing of E2-315.** N-terminal amino acid sequencing of purified protein was done using an Applied Biosystems 477A sequencer. Sample quantity provided for sequencing was estimated from absorbance of the purified protein at 280 nm.

■ **Solid phase immunoassays and RT-PCR.** For identification of GBV-C E2-315-containing fractions resulting from purification, a microtitre plate ELISA was performed. Serial dilutions of fractions were made in 0.1 M sodium phosphate buffer, pH 7.5 and coated onto microtitre plates at 60 °C for 2 h. This was followed by a blocking step with 5% (w/v) BSA in 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 at 40 °C for 1 h, followed by a 1 h 40 °C incubation with rabbit 10363 antiserum diluted 1:300 into diluent described previously (Dawson, *et al.*, 1992). Detection was with horseradish peroxidase-labelled goat anti-rabbit (H + L) IgG diluted to 0.2 μ g/ml. Fractions were assayed in duplicate.

ELISA of human sera using purified CHO cell-expressed GBV-C E2-315 protein was performed as described elsewhere (Dille *et al.*, 1997). ELISA using *E. coli*-expressed CKS-GBV-C E2 fusion protein (E2-212) was performed as previously described (Dawson *et al.*, 1992, 1996) using an optimized coating concentration of 4 μ g/ml E2-212. Samples were assayed in duplicate.

Details of RT-PCR on human serum samples were as described previously (Dawson *et al.*, 1996). Two PCR reactions, which used non-overlapping sets of primers, were performed on each serum sample. One set of primers was degenerate and based on sequences in the NS3 region of GBV-C, whereas the other set was based on sequences in the 5'-untranslated region. A serum sample was considered positive for GBV-C RNA if products of the expected size were obtained in both PCR reactions.

■ **Semliki Forest virus (SFV) expression of GBV-C E2-336 protein.** A related 336 amino acid fragment of the GBV-C E2 protein, representing amino acids 204–539 of the West African isolate GBV-C polypeptide and referred to as E2-336, was expressed in a transient expression system using the Semliki Forest virus pSFV1 vector and the BHK-21 cell line as described previously (Pilot-Matias *et al.*, 1996b). Radioimmunoprecipitation assay (RIPA) involved metabolic labelling of transfected cells using [³⁵S]cysteine and precipitation of labelled protein using human sera as described previously (Pilot-Matias *et al.*, 1996b).

Results

Expression of GBV-C E2 protein

Expression of E2-315 within CHO cells was detected by immunofluorescence staining using rabbit antiserum 10363 (Fig. 2A) and human sera (Fig. 2C). Perinuclear fluorescence was observed, suggesting localization of protein within the endoplasmic reticulum, which is consistent with E2-315 being secreted. No fluorescence was seen with the negative control cell line (Fig. 2B, D). Secreted E2-315 protein was detected by Western blotting using FLAG M2 monoclonal antibody and 10363 and 10365 rabbit antisera (Fig. 3). Secreted E2 was of molecular mass approximately 48–56 kDa and migrated as a diffuse band, suggesting it was a glycoprotein; the predicted

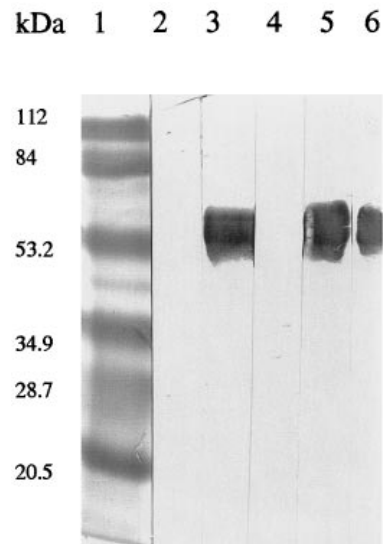


Fig. 3. Western blot analysis of CHO cell-expressed GBV-C E2-315. Protein in VAS medium harvested from CHO cells transfected with GBV-C E2-315 expression plasmid was electroblotted onto ProBlott membrane after separation of denatured proteins by 12% SDS-PAGE. The blot was probed with antibody as described in Methods. Lane 1, molecular mass markers in kDa as indicated; lane 2, negative control protein expression probed with rabbit 10363 antiserum; lane 3, GBV-C E2-315 expression probed with rabbit 10363 antiserum; lane 4, negative control protein expression probed with rabbit 10365 antiserum; lane 5, GBV-C E2-315 expression probed with rabbit 10365 antiserum; lane 6, GBV-C E2-315 expression probed with FLAG monoclonal antibody.

molecular mass of the polypeptide chain of E2-315 is 35 kDa. Since the FLAG sequence is at the C terminus of the protein, detection with FLAG monoclonal antibody also confirmed the presence of full-length recombinant protein.

Purification of GBV-C E2 protein

Purification of GBV-C E2-315 was accomplished by affinity chromatography using FLAG M2 monoclonal antibody. Fig. 4(A) shows a Coomassie Brilliant Blue R-250-stained SDS-polyacrylamide gel of the purification. Scanning densitometry of gel lanes containing purified GBV-C E2-315 protein showed it to be greater than 90% pure (data not shown). Western blotting (Fig. 4B) and microtitre plate ELISA (data not shown) demonstrated that E2-315 protein bound to the column and was eluted with FLAG peptide. No further E2-315 was eluted with a more stringent 0.1 M glycine-HCl, pH 3.5 wash.

Biochemical characterization of purified E2-315

Purified GBV-C E2-315 was deglycosylated under both denaturing and native conditions by N-Glycanase. Under denaturing conditions, the product had a molecular mass of 35 kDa (Fig. 5, lane 3). This is the predicted size for the E2-315 polypeptide chain. Deglycosylation under native conditions

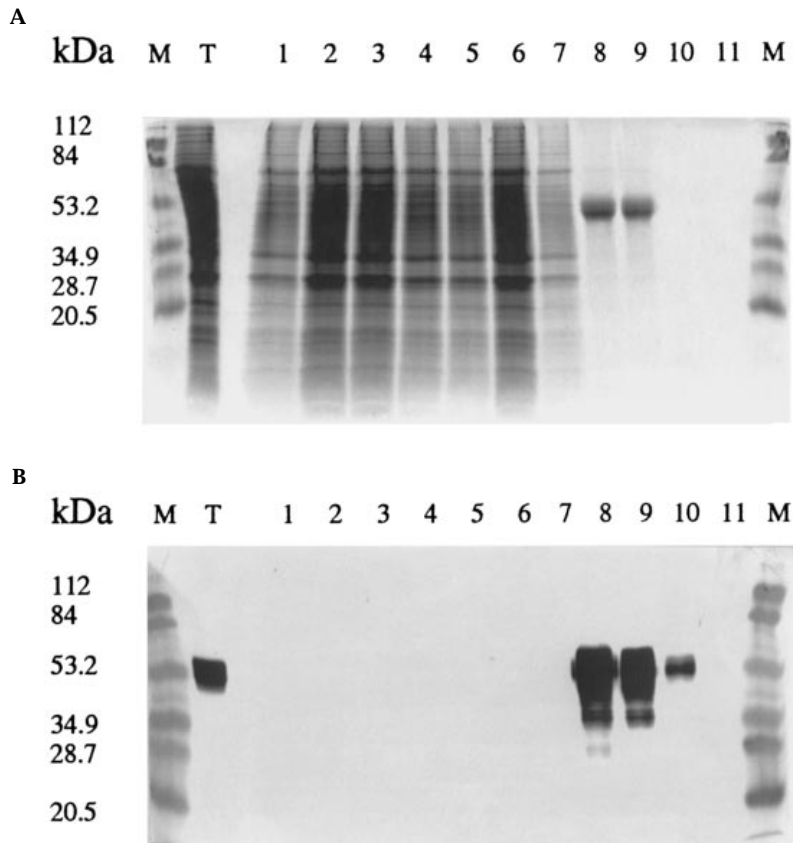


Fig. 4. Purification of GBV-C E2-315 using FLAG M2 monoclonal antibody affinity chromatography. (A) Coomassie Brilliant Blue staining of denatured protein from fractions analysed by 12% SDS-PAGE as described in Methods: M, molecular mass markers in kDa as indicated; T, protein loaded onto the affinity column; 1–7, sequential fractions of non-bound protein which eluted in the column buffer; 8–11 sequential fractions of bound protein which was eluted with FLAG peptide. (B) Western blot analysis using rabbit 10363 antiserum: lane identification as for (A); molecular masses of markers are indicated in kDa on the left.

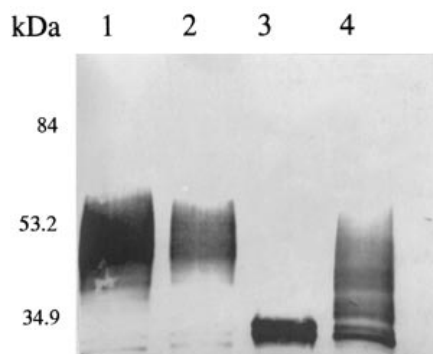


Fig. 5. Deglycosylation of purified GBV-C E2-315 using N-Glycanase. Deglycosylation and control incubations were performed as described in Methods and assessed using Western blot analysis with FLAG M2 monoclonal antibody. Lane 1, unincubated sample; lane 2, denatured sample incubated in the absence of N-Glycanase; lane 3, denatured sample incubated in the presence of N-Glycanase; lane 4, native sample incubated in the presence of N-Glycanase. Positions of molecular mass markers in kDa are indicated on the left.

required more enzyme and was not as complete (Fig. 5, lane 4), suggesting that some of the *N*-glycosylation sites were inaccessible to N-Glycanase in the native protein conformation.

N-terminal amino acid sequencing of purified E2-315 confirmed the presence of the N-terminal sequence GAPASV

of GBV-C E2. Identification of the purified protein as E2-315 was further supported by its reactivity on Western blot with rabbit E2 peptide antiserum 10365 (data not shown).

Reactivity with human sera

ELISA using CHO cell-expressed GBV-C E2-315 demonstrated that antibodies to GBV-C E2 protein were detected in commercial plasma donors, injection drug users and patients with non-A–E hepatitis (Table 1). Nineteen out of 28 samples were positive for E2 antibodies. Five out of 28 samples were GBV-C RT-PCR positive and of these only two were E2 antibody-positive. Thus, 17/19 E2 antibody-positive samples were RT-PCR negative and 3/5 RT-PCR positive samples were E2 antibody-negative.

All 19 human sera detected as E2 antibody-positive by E2-315 ELISA were positive by RIPA using the transiently expressed GBV-C E2-336 protein (Table 1). Samples with a strong signal in ELISA gave similar results in RIPA. An additional sample (number 22 in Table 1) that was positive by RIPA but negative in the ELISA was positive by IFA of CHO cells expressing the E2-315 protein.

Sixteen of 19 samples which were E2 antibody-positive by E2-315 ELISA were also positive by IFA (Table 1, Fig. 2). As noted above, sample number 22 was positive by IFA and RIPA but negative by E2-315 ELISA. Seventeen of 20 E2-336 RIPA

Table 1. Characterization of human sera reactivity with GBV-C E2 proteins

Samples 1–13 include sera from commercial plasma donors, injection drug users and others considered 'at risk' for exposure to GBV-C. Samples 14–23 are sera from individuals with non-A–E hepatitis. Samples 24–28 are sera from volunteer blood donors. For ELISA, + encompasses samples giving sample to negative control ratios (S/Ns) of 7.5–50, ++ encompasses samples giving S/Ns of 5–100, +++ encompasses samples giving S/Ns of > 100. The negative cut-off value for ELISA was determined as described previously (Dille *et al.*, 1997).

Sample	PCR status	Western blot (denatured E2-315)	ELISA reactivity (E2-315)	IFA reactivity (E2-315)	RIPA (E2-336)
1	+	–	–	–	–
2	+	–	–	–	–
3	+	–	–	–	–
4	–	–	+	–	++
5	–	–	+	–	++
6	–	+	+	++	++
7	+	–	++	++	+++
8	+	+	++	+	+++
9	–	+	+++	+++	+++
10	–	–	+++	+	+++
11	–	–	+	+	+++
12	–	–	+	+	+++
13	–	–	++	+++	+++
14	–	–	+	+	+++
15	–	–	+	+	+++
16	–	+	+++	+++	+++
17	–	–	+	+++	+
18	–	–	+	–	+
19	–	–	++	+	+++
20	–	+	+++	+++	+++
21	–	+	+++	++	+++
22	–	–	–	++	++
23	–	–	+++	+	+++
24	–	–	–	–	–
25	–	–	–	–	–
26	–	–	–	–	–
27	–	–	–	–	–
28	–	–	–	–	–

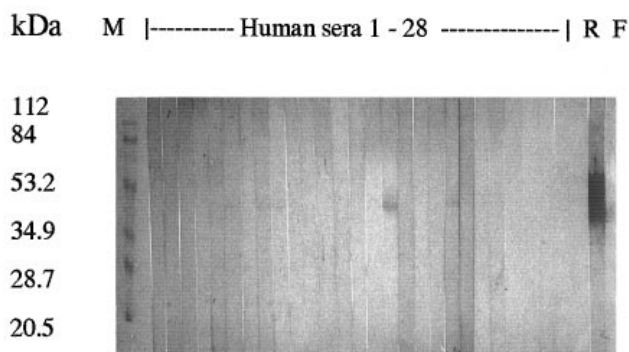


Fig. 6. Western blot analysis of human sera reactivity with purified denatured GBV-C E2-315. Purified E2-315 protein was denatured and run on a 12% SDS-PAGE gel and Western blotted as described in Methods. The blot was cut into strips and reacted with human sera samples 1–28, rabbit 10363 antiserum (R) and FLAG M2 monoclonal antibody (F). Molecular masses of markers (M) are indicated in kDa on the left.

positives were E2-315 IFA positive. None of the samples which were non-reactive by E2-315 ELISA or E2-336 RIPA were positive in IFA.

Of the 19 samples which were positive in the E2-315 ELISA, only six were positive by Western blot analysis, though weakly so (Table 1 and Fig. 6). None of the samples which were non-reactive in the E2-315 ELISA were positive by Western blot. The Western blot results were confirmed by dot blot in which native and denatured E2-315 were probed with the 28 human sera. Results for 12 representative sera are shown in Fig. 7. Native and denatured E2-315 gave the same reactivities as seen in E2-315 ELISA and Western blot, respectively. Furthermore, in an ELISA based on a denatured *E. coli*-expressed CKS-GBV-C E2 fusion (E2-212), only two E2 antibody positives were detected amongst the 28 human sera tested (data not shown).

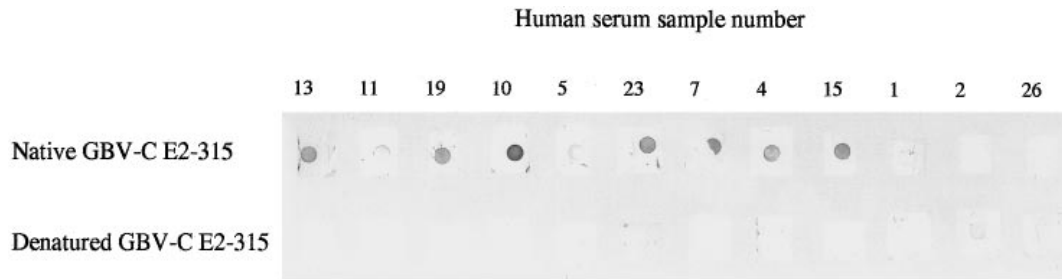


Fig. 7. Dot blot analysis of human sera reactivity with purified native and denatured GBV-C E2-315. Dot blotting was performed as described in Methods. Human sera sample numbers are as in Table 1 and Fig. 6.

Discussion

Glycosylated GBV-C E2-315 protein was expressed and secreted from a stable CHO cell line. The inclusion of the FLAG sequence at the C terminus of GBV-C E2-315 allowed for a one-step affinity purification of the recombinant protein with minimal losses. Reactivity with E2 10365 antiserum and N-terminal protein sequencing confirmed that the proper protein was being expressed. As designed, truncation at the C terminus to remove the hydrophobic putative membrane-anchoring domain appeared to allow the recombinant E2-315 to be secreted. Such membrane-bound sequences are unlikely to be involved in folding interactions with the rest of the protein, so one might predict that the recombinant protein could be conformationally similar to native viral E2. The observed reactivity of recombinant E2-315 with antibodies in human sera supports that this is indeed so.

In support of our previous studies (Pilot-Matias *et al.*, 1996*b*; Dille *et al.*, 1997), the E2-315 ELISA data presented here indicate that the prevalence of GBV-C is higher than that observed by detection of GBV-C RNA alone. This observation has been made both with specimens obtained from individuals considered 'at risk' for GBV-C infection and among individuals with non-A-E hepatitis. For HCV infections, there is a high degree of correlation between the detection of antibodies to HCV E2 and the presence of HCV RNA detected by RT-PCR (Zaaijer *et al.*, 1994; Lesniewski *et al.*, 1995). In contrast, for GBV-C infections, there is a negative correlation between the presence of antibodies to GBV-C E2 protein and GBV-C RNA as detected by RT-PCR, as noted in previous studies (Pilot-Matias *et al.*, 1996*b*; Dille *et al.*, 1997). Thus, it is possible that GBV-C E2 antibodies may be involved in serum virus clearance, as is noted for antibodies directed against E2 proteins of several flaviviruses and pestiviruses (Donis *et al.*, 1988; Weiland *et al.*, 1990; Rumenapf *et al.*, 1991; Monath & Heinz, 1996). However, further studies are needed to confirm this and for now it is clear only that a temporal association exists between serum virus clearance and appearance of antibodies to GBV-C E2 protein. A further similarity between GBV-C and several flavi- and pestiviruses studied thus far (Lewis *et al.*, 1993; Becher *et al.*, 1994) is that there is little

variation in GBV-C E2 sequences amongst different genotypes (Erker *et al.*, 1996). Again, this is in contrast to HCV, which possesses a hypervariable region in the E2 protein, believed to contribute to the ability of the virus to escape immune clearance (Farci *et al.*, 1992; Weiner *et al.*, 1992). While the E2 proteins of HCV and GBV-C differ with respect to their heterogeneity, both viruses exhibit the ability to establish persistent infections in their hosts which may last for many years (Alter *et al.*, 1992, 1997*a, b*; Linnen *et al.*, 1996; Dawson *et al.*, 1996; Masuko *et al.*, 1996). Since the persistence of GBV-C does not appear to rely on hypervariable regions within E2 it may be related to the ability of the virus to evade immune control by other means, perhaps through one of several mechanisms recently elucidated for other viruses which persist in the host (reviewed by Paroli *et al.*, 1995), some of which involve interference by virus with T cell responses that normally clear infection. It is noteworthy that no consistent antibody response to any one GBV-C protein has yet been found in individuals positive for serum GBV-C RNA (Pilot-Matias *et al.*, 1996*a*; Dawson *et al.*, 1996). For HCV, chronic infection occurs in 50–80% of individuals following acute infection (reviewed in Dawson *et al.*, 1995). The persistence of GBV-C viral RNA, as detected by RT-PCR, suggests GBV-C infection can last for many years (as long as 16 and maybe longer) in some individuals, whereas others clear the virus within a year or two or less (Linnen *et al.*, 1996; Dawson *et al.*, 1996; Pilot-Matias *et al.*, 1996*b*; Masuko *et al.*, 1996; Dille *et al.*, 1997; Alter *et al.*, 1997*a, b*). Our present and previous (Pilot-Matias *et al.*, 1996*b*; Dille *et al.*, 1997) data demonstrating the presence of E2 antibody in the absence of GBV-C RNA suggest that many individuals can and do clear the virus; in fact, prevalence of GBV-C E2 antibody appears to be greater than that of GBV-C RNA. However, since relatively little is yet known regarding the persistence of E2 antibody or of GBV-C viraemia, further studies with more serial human serum samples are needed to better estimate the persistence, both in terms of likelihood and longevity, of this recently discovered virus versus its ability to be cleared.

Good correlation of E2-315 IFA with E2-315 ELISA results, together with the observation of strong IFA reactivities with human sera, implies that, antigenically, intracellular E2-315 is

very similar to secreted E2-315. Since intracellular E2 is presumably a population of molecules, some only partially synthesized, folded and glycosylated, others fully so, this correlation could imply that GBV-C E2 folds quickly in the endoplasmic reticulum to a native-like structure. Relatively fast folding of the related HCV E2 in the endoplasmic reticulum has in fact been demonstrated (Dubuisson & Rice, 1996).

The native CHO cell-expressed GBV-C E2-315 ELISA was more effective at detecting human E2 antibodies than any detection system based on denatured GBV-C E2 protein (Western or dot blot using denatured E2-315 or ELISA based on denatured *E. coli*-expressed E2-212 fusion protein). The results imply that conformational epitopes on the E2 protein are important in the human immune response to GBV-C infection. A similar predominant immune response to conformational epitopes on envelope proteins is seen for HCV, as well as for other flaviviruses and pestiviruses (Nowak & Wengler, 1987; Guirakhoo *et al.*, 1989; Hall *et al.*, 1990; Weiland *et al.*, 1992; Chien *et al.*, 1993; Lesniewski *et al.*, 1995; Harada *et al.*, 1995). Clearly, the data demonstrate the usefulness of an ELISA based on native glycosylated GBV-C E2 for investigation of GBV-C exposure.

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