Recombinant human monoclonal antibodies against different conformational epitopes of the E2 envelope glycoprotein of hepatitis C virus that inhibit its interaction with CD81

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Introduction

Infection with hepatitis C virus (HCV) is a global health problem, with an estimated 1–2% of the world’s population chronically infected. Long term complications may be severe, and include liver failure and hepatocellular carcinoma. Interferon-α is the most effective pharmaceutical agent for treating the infection, with a success rate of 20–25%. Recently, combination therapy with Ribavirin has been reported to improve the rate of complete sustained response to about 40% (Reichard et al., 1998).

Infection by HCV induces a broad cell-mediated immune response as well as a humoral response (Alter et al., 1989; Rehermann et al., 1996). Several reports indicate that elicited antibodies may neutralize the infectivity of the virus (Farci et al., 1994; Shimizu et al., 1994). Despite this, most infected individuals develop a chronic infection. Although antibodies elicited by natural infection are not sufficient to clear the infection or even to prevent reinfection, several reports indicate that the antibody response to HCV plays an important role in controlling the infection and modulating its course. HCV-infected patients with agammaglobulinaemia have a much more rapidly progressing disease than other patients with hepatitis C (Bjørø et al., 1994), and passively transfused antibodies to HCV have been shown to reduce the risk of HCV reinfection after liver transplantation (Feray et al., 1998). In chimpanzee experiments, repeated post-exposure treatment with polyclonal anti-HCV antibodies suppressed all signs of infection and liver disease for as long as antibodies were administered (Krawczynski et al., 1996).

The lack of a reliable cell culture system for HCV has made studies of virus neutralization difficult. Two putative targets for neutralizing antibodies in the E2 envelope glycoprotein...
have been suggested. The first is the hypervariable region (HVR) located in the N terminus of E2. This has been shown to encode linear epitopes that change considerably over time, resulting in new variants that escape the reactivity of previously produced, specific antibodies (Kato et al., 1993). Several reports indicate that antibodies to HVR may neutralize the virus (Farcă et al., 1996; Shimizu et al., 1996; Zibert et al., 1995). During acute HCV infection, early appearance of antibodies to the HVR predicts clearance of the infection (Allander et al., 1997; Zibert et al., 1997).

Secondly, E2 glycoprotein interacts with CD81 on human cells (Pileri et al., 1998). The capacity of antibodies to inhibit this interaction can be assessed by a competition assay, referred to as the neutralization of binding (NOB) assay. The NOB mediating site appears to be different from HVR (Rosa et al., 1996). High NOB titres were shown to correlate with protection in vaccinated chimpanzees. Furthermore, patients from a rare group that spontaneously clear chronic HCV infection were found to have high NOB titres (Ishii et al., 1998).

Neutralizing antibodies of human origin could be considered for use in passive immunotherapy, in particular if broadly reactive against different strains of the virus. Preferably, such antibodies would be monoclonal in order to predict the effects in vivo; however, a combination of several monoclonal antibodies may be superior in the case of a variable virus such as HCV. Secondly, generation of monoclonal antibodies is the most useful approach to study and characterize the immune response to the virus, provided that the antibodies isolated originate from an infected donor (Chanock et al., 1993); indeed, a recent report has applied this to the study of human anti-HCV antibodies (Burioni et al., 1998).

In the present report, we describe the isolation of human, monoclonal anti-HCV antibodies from a combinatorial Fab-library derived from an HCV-infected individual. The experiment was designed to result in cross-genotype reactive antibodies to the E2 envelope glycoprotein. Initially isolated as Fab clones, they were eventually expressed as IgG1 molecules in eukaryotic cells, and tested for functional anti-HCV properties.

**Methods**

**Antigens.** Recombinant E2 protein of genotype 1a and 1b, expressed in CHO cells, or a similarly expressed E1/E2 heterodimer protein (genotype 1a) were used for selection and characterization of the antibodies. The expression and purification of these recombinant proteins have been reported elsewhere (Rosa et al., 1996; Spaete et al., 1992).

**Bone marrow donor.** The bone marrow was obtained from a 60-year-old male deferred blood donor, deferred when found HCV positive at the implementation of blood donor screening for anti-HCV. He had no symptoms of the infection, and the time and source of infection are unknown. He had received no antiviral treatment for the infection. He had been viraemic for several years as determined by retrospective analyses on stored sera. The HCV genotype in serum at the time of bone marrow donation was found to be 2b (Widell et al., 1994) by PCR-based typing.

**Library construction.** Lymphocytes were isolated from the bone marrow sample using Ficoll–Paque (Pharmacia). Total RNA was extracted by the acid guanidinium thiocyanate–phenol method (Chomczynski & Sacchi, 1987). First strand cDNA synthesis utilizing oligo(dT) priming of 10 µg of RNA was performed (cDNA synthesis kit) and heavy (Fh) and light chain DNAs were PCR amplified using 5’ biotinylated primers of previously published sequences for y1 and k chains (Kang et al., 1991; Persson et al., 1991). The PCR products encoding the heavy and light chains were pooled separately, phenol–chloroform extracted and ethanol precipitated.

Twelve µg each of heavy and light chain DNA were gel-purified on a 2.5% agarose gel, electroeluted, and digested with the restriction endonucleases XhoI/SpeI and SacI/XhoI, respectively (Life Technologies). The digested PCR products were subsequently gel-purified, recovered by electroelution, and ligated into the vector pComb3H after it had been digested with the corresponding enzymes and gel-purified/electroeluted as described previously for a similar vector system (Barbas et al., 1991; Barbas & Wagner, 1995). The resulting combinatorial library was expressed in phage, including harvesting of phage, as reported by Samuelsson et al. (1995).

**Selection procedure.** Four wells of a microtitre plate (Costar 3690) were coated at 4 °C overnight with 50 µl of purified recombinant E2 protein (genotype 1a), at 2.5 µg/ml, or a similarly expressed and purified E1/E2 heterodimer protein (genotype 1a) at 10 µg/ml (Spaete et al., 1992). The wells were blocked by filling completely with 5% non-fat dry milk in PBS for 1 h at 22 °C. Phage library (50 µl, 5 × 10¹⁵ c.f.u.) was then added to each well. Adsorption of phage, washing, and elution of phage was performed as described by Burton et al. (1991). Washing was done one to ten times as described below. E. coli XL-1 blue was infected with the eluted phage, an aliquot was plated, and phage was propagated after each round of panning as described by Samuelsson et al. (1995).

Three separate panning experiments were performed. In the first experiment (panning series I), three rounds of panning with recombinant E2 protein were performed, and the number of washes was increased for each round (one, three and ten washes respectively). The second experiment (panning series II) consisted of a single selection with ten washes, using the same antigen. Panning series III was performed as series I, but utilizing the recombinant E1/E2 protein heterodimer.

**Expression of Fab in E. coli.** Fab fragments were expressed by growing ampicillin-resistant E. coli colonies containing the Fab plasmid (with the phage-derived gll part intact, or deleted by digestion with SpeI and XhoI) in SB with 50 µg/ml ampicillin and 1% glucose, until OD₆₀₀ = 1.0 was reached. Bacteria were pelleted by centrifugation, and media exchanged to SB with 1 mM IPTG and 20 mM MgCl₂; bacteria were then resuspended and the culture incubated at room temperature on a shaker platform at 290 r.p.m. overnight. The following day, cells were spun down, the supernatant discarded, PBS added (2% of original culture volume) and the periplasmic content released by freeze–thawing three times. Released bacterial DNA was digested with DNase I, bacterial debris pelleted by centrifugation, and the Fab-containing supernatant aliquoted to new vials. The material was kept at −20 °C until used.

**Expression of Fab and IgG in CHO cells.** Seven clones (L1, L3, 1:5, 1:7, 1:11, A8 and A12) were expressed as Fab fragments and whole IgG1 in CHO cells. The genes encoding heavy and light chains for each clone were transferred from pComb3H to the vectors pLC162 and pdgG1, transfected into CHO cells by using Lipofectamine (Life Technologies) according to the manufacturer’s instructions, and expressed as detailed by Samuelsson et al. (1996). Antibodies were
secreted into the medium, and cell culture supernatant was collected and stored in aliquots in $-20\,^\circ\text{C}$. The unpurified supernatant was used for most of the subsequent assays. However, for epitope mapping studies and the NOB assay, IgG was further purified from the culture supernatant on Protein G columns (Amersham Pharmacia).

ELISA for quantification of Fab and IgG. The concentrations of expressed Fab and IgG were determined by ELISA as previously described (Samuelsson et al., 1995, 1996).

ELISA for anti-HCV E2 and E1/E2 reactivity. Recombinant E2 (genotype 1a or 1b) or the E1/E2 heterodimer (genotype 1a) was diluted to 1 $\mu$g/ml in 0.05 M carbonate–bicarbonate buffer, pH 9.6, and coated to microtitre wells (Costar 3690) overnight at 4 $^\circ\text{C}$. Unbound antigen was discarded, and the wells were blocked with 5% non-fat dry milk in PBS for 60 min at room temperature. Blocking solution was discarded, and antibody solutions to be tested added in 1:1, 1:10 and 1:100 dilutions (diluent: PBS with 0.1% NP-40). The plates were incubated at 37 $^\circ\text{C}$ for 2 h and washed five times with PBS with 0.05% Tween 20 (PBS-T); alkaline phosphatase (AP)-coupled goat anti-human F(ab)$^3$ (Pierce) or $\alpha$-anti-human IgG (Fc) (ANL produkter, Alvsjö, Sweden) antibodies in a 1:1000 dilution were then added. After 60 min at 37 $^\circ\text{C}$ and subsequent washes, substrate solution ($p$-nitrophenyl phosphate, Sigma) was added and absorbance measured at 405 nm. Cut-off value for positive readings was deemed to be four times the absorbance value obtained for a negative control sample, an anti-HIV Fab or IgG of equal concentrations (Burton et al., 1991; Samuelsson et al., 1996). For control purposes, BSA (Sigma), HIV gp120/LA (Intracell, Cambridge, MA, USA) and tetanus toxoid (SBL Vaccin, Solna, Sweden) coated at 1 $\mu$g/ml were used in corresponding ELISAs to control for unspecific reactivity.

Sequencing of Fab clones. Sequencing of the Fab clones was performed utilizing fluorescent-labelled primers [SEQKb (5’ ATA GAA GTT CCT AGC GCC ATT GCA G) for $\kappa$ light chains, and SEQGb (5’ GTC GTT GAC CAG GCA GCC CAG) and pel-seq (5’ ACC TAT TGC CTA CCG CAG CCG) for $\gamma$ heavy chains] and an ALF automated sequencer (Pharmacia Biotech).

Inhibition ELISA for affinity determination. The affinity of the Fab and IgG molecules for the E2 antigens was estimated using an inhibition ELISA (Persson et al., 1991; Rath et al., 1988). Samples to be tested were first titrated at 10-fold dilutions in order to find a suitable concentration from which 10-fold reduced concentration gave a substantial reduction in detected binding in the anti-E2 ELISA (as above). Coating of microtitre wells with E2 antigen and subsequent blocking were done as described for the anti-E2 ELISA. Fab and IgG samples, at appropriate dilutions, with or without added soluble E2 (at tenfold dilutions, maximum concentration 5 $\mu$g/ml) were added to the wells, and incubated at 37 $^\circ\text{C}$ for 2 h. The plates were washed uniformly four times with PBS-T, and bound antibody was detected using AP--streptavidin or AP--anti-Fc, as described above for ELISA. The reduction in absorbance in the presence of soluble E2 was calculated and the concentration needed for a 50% reduction estimated by extrapolation.

Cross-competition ELISA. A competitive ELISA was used to determine whether the cloned antibodies shared the same epitopes. Recombinant E1/E2 (genotype 1a) protein was used as antigen and experimental conditions were as described above for the anti-E2 ELISA. Clone L1 IgG was biotinylated using EZ-Link Biotin-LC-Hydrazide (Pierce) according to the manufacturer’s instructions. Equal volumes of the biotinylated L1-IgG at approximately 0.06 $\mu$g/ml and IgG of one of the other clones, at approximately 0.1 and 0.025 $\mu$g/ml, were mixed and added to duplicate wells. After a 2 h incubation at 37 $^\circ\text{C}$ and subsequent washes, AP--streptavidin (Pierce) was added for 1 h, the plate washed, and substrate added. The negative control competitor was a human monoclonal IgG1 antibody (Gp13) directed against HIV-1 gp120 (Shutten et al., 1993).

Inhibition of E2 binding to cells. E. coli-produced Fab proteins, as well as Fab and Protein G-purified IgG1 expressed in eukaryotic cells, were tested for their capacity to block the binding of recombinant E2 to MOLT-4 cells. This was assessed by flow cytometry using the NOB-assay as detailed elsewhere by Rosa et al. (1996).

Results

Library size

The ligation of $\kappa$ light chain genes into pComb3H gave a library of $2 \times 10^7$ members. The subsequent ligation of $\gamma$ Fd genes into the light chain library resulted in a combinatorial library with $2 \times 10^6$ members.

Isolation of E2 reactive clones

After selection by panning, clones were initially screened for E2 reactivity and promptly sequenced, in order to identify multiple copies of the same original clone. In panning series I, a 100-fold increase in eluted phage was noted after three rounds of selection. Ten clones were assayed (L1–L10), and all were found to be strongly reactive with the E2 antigen with no cross-reactions to non-HCV antigens. Surprisingly, all of them carried very similar CDR3 sequences in their heavy chains (the H3 region), indicating that they were derived from the same B-cell clone (Litwin & Shlomchik, 1990). However, while the VDJ junctions and the length of the H3 region were identical, they did show a number of different point mutations in their heavy chains, and were combined with different light chains (data not shown).

To isolate additional clones, another, single panning was performed (series II). From this experiment, of 20 clones initially tested, ten produced sufficient amounts of anti-E2 specific Fab. Sequencing showed that seven carried heavy chains related to the ones found in the series I panning (the L1–L10 clones). Three clones had H3 regions that were distinct from the L-clones (1:5, 1:7, 1:11). However, clones 1:5 and 1:11 had genetically related H3 regions.

A third selection experiment (panning series III) was performed using the E1/E2 heterodimer antigen. No significant difference in eluted phage was observed. After three rounds of panning, 30 clones were assayed, of which 16 were E1/E2 reactive. All 16 clones were also reactive with E2 alone. Twelve clones had an H3 sequence similar to L1–L10, while four of the clones had unique H3 sequences. Two of the clones were selected for further analysis because they had a higher affinity to E2 (clones A8 and A12).

In summary, of 50 clones tested from series I–III, 36 were E2 specific, and 29 shared a related heavy chain. The deduced amino acid sequences of the variable regions of seven clones
(a)

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Fig. 1. Deduced amino acid sequences of the variable light (a) and heavy (b) chain regions for seven clones (four carrying unique heavy chains). The CDR regions as suggested by Kabat et al. (1991) are underlined.

(b)

The antibodies reacted with the HCV antigens in ELISA with a higher reactivity to the E1/E2 complex compared to E2 only, but not to control antigens (Fig. 2). Clones 1:7, A8 and A12 reacted with E2 derived from HCV of both genotype 1a and 1b in ELISA.
Human monoclonal antibodies to HCV

Clone ELISA reading (A405)

Fig. 2. ELISA reactivity for seven Fab clones with recombinant E2 protein of genotype 1a and 1b, E1/E2 protein complex (genotype 1a), and control antigens (BSA and HIV-1 gp120). The bars indicate the mean absorbance at 405 nm.

No difference in antigen-specific reactivity in ELISA was seen when comparing Fab molecules with the Fd part expressed as a fusion protein to cpIII of phage, or as an ordinary Fab (Fd \( \rightarrow \) LC) or when comparing bacterially expressed Fab with Fab or IgG of the same clone expressed in CHO cells (data not shown).

Expression levels

In *E. coli*, most Fab clones were found to produce 0.2–2.0 mg Fab/1 of culture, corresponding to 10–100 \( \mu \text{g/ml} \) in the periplasmic preparations. In CHO cell culture supernatants, the yield was between 0.1 and 0.5 \( \mu \text{g/ml} \) for Fabs and 0.5–1.0 \( \mu \text{g/ml} \) for IgG. After Protein G affinity purification of IgG1, concentrations of 10–40 \( \mu \text{g/ml} \) were obtained in the preparations.

Detection of heavy and light chains by Western blot

To test for correct expression of both chains, the IgG proteins were analysed in Western blot for each of the five clones expressed in CHO cells, using antiserum for human Fc- and light chains. Both chains were approximately equally well-expressed in all clones (data not shown). Furthermore, the expression of Fd- and light chains in four bacterially expressed clones was investigated (L1, L3, A8, A12), and both chains were detected. The Fd-chain expressed in fusion with the truncated phage protein cpIII showed an approximate molecular mass of 70 kDa (data not shown).

Reactivity with the E2 protein in Western blot

None of the seven Fab proteins tested reacted with E2 of genotype 1a in the Western blot, but serum from the genotype 2b-infected bone marrow donor, drawn at the same time as the bone marrow aspiration, did react with the denatured E2 protein. However, when the antigen was gel-separated under non-denaturing conditions, the two clones (1:7 and A8) tested bound the native E2 protein on the nitrocellulose strip under otherwise identical assay conditions.

Affinity for the E2 protein

The approximate affinities of the antibody clones for the recombinant E2 antigen of genotype 1a, as determined in an inhibition ELISA, varied between \(< 1 \times 10^7\) and \(2 \times 10^8\) \(\text{M}^{-1}\) (Table 1). Affinities were the same whether the antibodies were expressed as Fab fragments in *E. coli*, or Fab fragments or IgG in CHO cells. In addition, the three clones that reacted with the

Table 1. Approximate affinities for recombinant E2 glycoprotein (genotype 1a) of the antibody clones expressed as IgG in CHO cells or Fab in *E. coli*

<table>
<thead>
<tr>
<th>Clone</th>
<th>IgG</th>
<th>Fab</th>
</tr>
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<tbody>
<tr>
<td>L1</td>
<td>(4 \times 10^7)</td>
<td>(4 \times 10^7)</td>
</tr>
<tr>
<td>L3</td>
<td>(4 \times 10^7)</td>
<td>(4 \times 10^7)</td>
</tr>
<tr>
<td>1:5</td>
<td>(&lt; 1 \times 10^7)</td>
<td>(&lt; 1 \times 10^7)</td>
</tr>
<tr>
<td>1:7</td>
<td>(2 \times 10^8)</td>
<td>(2 \times 10^8)*</td>
</tr>
<tr>
<td>1:11</td>
<td>(4 \times 10^7)</td>
<td>(4 \times 10^7)</td>
</tr>
<tr>
<td>A8</td>
<td>Not tested</td>
<td>(2 \times 10^8)*</td>
</tr>
<tr>
<td>A12</td>
<td>Not tested</td>
<td>(1 \times 10^8)*</td>
</tr>
</tbody>
</table>

* Affinity of Fab was also tested for E2 of genotype 1b, and results were similar to those obtained with E2 of genotype 1a.
E2 antigen of genotype 1b (1:7, A8, A12) had equal affinities for both genotypes.

**Epitope mapping**

Cross-competition findings for the seven clones investigated indicated that five clones competed for the same or overlapping epitopes as clone L1 (Fig. 3). Clone 1:7 did not compete for the ‘L1-epitope’, and clone A12 showed only a slight inhibition. These two clones must be assumed to bind to other parts of E2.

**Inhibition of E2 binding to cells**

Clones representing binding to distinct epitopes were tested as purified IgG1 for inhibition of E2 binding to target cells in the NOB assay (Rosa et al., 1996). All clones (L3, 1:7, A8, A12) tested efficiently inhibited the binding of recombinant E2 glycoprotein (type 1a) to MOLT-4 cells at a 50% inhibition concentration of 0.3–0.5 µg/ml. A human monoclonal IgG1 antibody (CL3, expressed and purified in the same manner) to HIV-1 envelope glycoprotein gp120 was negative (Table 2a).

All seven clones characterized were also tested for blocking of E2 (both genotype 1a and 1b) as E. coli-expressed, non-purified periplasmic preparations of Fabs (clones L1, L3, 1:5, 1:7, 1:11, A8 and A12). They were all positive for NOB activity, even at a 10–100-fold lower concentration than found for the purified IgG1; clones A8, 1:7, L1 and L3 had very high NOB activities (Table 2b). Two control anti-HIV gp120 Fab clones had no NOB activity (Table 2b).

**Discussion**

In the present report we describe the isolation of 36 E2-specific antibody clones from a patient chronically infected by HCV. The choice of a chronically infected donor, who is obviously not protected from HCV infection, for this type of experiment may seem irrational. However, experimental evidence suggests that protective antibodies are present during chronic infection (Farci et al., 1994), and indeed, generation of antibodies to HIV-1 using exactly the same approach has resulted in antibody clones having excellent neutralizing activity, including protection from infection in an animal model (Burton et al., 1994; Gauduin et al., 1997). The donor was infected with HCV genotype 2b, and the antigens used for selection were genotype 1a. This approach selects for antibodies directed to conserved epitopes which are supposedly of greater interest for development of vaccine and immunotherapy (Burioni et al., 1998). On the other hand, antibodies directed to genotype-specific or strain-specific epitopes will be lost. Whether strain-specific antibodies are necessary for protection is unknown. Some reports suggest that this may be

### Table 2. NOB titres of anti-E2 antibody clones

The Fab or IgG concentration (in µg/ml) needed to block the binding of E2 by 50% is given (NOB<sub>50</sub>) (Rosa et al., 1996).

(a) Blocking of E2 glycoprotein (genotype 1a) binding to target cells by purified IgG1 tested for four antibody clones specific for distinct epitopes

<table>
<thead>
<tr>
<th>IgG1 clone</th>
<th>NOB&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:7</td>
<td>0.5</td>
</tr>
<tr>
<td>L3</td>
<td>0.3</td>
</tr>
<tr>
<td>A8</td>
<td>0.4</td>
</tr>
<tr>
<td>A12</td>
<td>0.3</td>
</tr>
<tr>
<td>CL3</td>
<td>&gt; 10</td>
</tr>
</tbody>
</table>

* CL3 is an anti-HIV-1 directed human monoclonal antibody (IgG1) (Samuelsson et al., 1996).

(b) Fab clones tested as non-purified periplasmic preparations, with E2 antigen of both genotype 1a and 1b

<table>
<thead>
<tr>
<th>Fab clone</th>
<th>NOB&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E2 1a</td>
</tr>
<tr>
<td>1:5</td>
<td>2.5</td>
</tr>
<tr>
<td>1:7</td>
<td>0.01</td>
</tr>
<tr>
<td>1:11</td>
<td>0.1</td>
</tr>
<tr>
<td>L1</td>
<td>0.03</td>
</tr>
<tr>
<td>L3</td>
<td>0.02</td>
</tr>
<tr>
<td>A8</td>
<td>0.001</td>
</tr>
<tr>
<td>A12</td>
<td>0.1</td>
</tr>
<tr>
<td>b12&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>b14&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&gt; 10</td>
</tr>
</tbody>
</table>

* b12 and b14 are anti-HIV-1 directed human monoclonal antibodies (Fab, non-purified periplasmic preparations (Burton et al., 1991).
the case (Farci et al., 1994), while others point in the opposite direction (Feray et al., 1998; Ishii et al., 1998; Rosa et al., 1996; Scarselli et al., 1995).

Twenty-nine of the clones shared related heavy chains, indicating that they represent different stages of maturation of a single original B-cell clone. The striking dominance of this specificity may be due to a methodological bias, e.g. local predominance of a population of related B-lymphocytes at the site of bone marrow aspiration, or efficient maintenance of this particular sequence in E. coli. However, it is also possible that the majority of serum antibodies to E2 share these characteristics. Thus, the epitope to which they are directed may well be an immunodominant epitope.

Seven antibody clones representing different heavy chains were analysed in detail. Five of the clones were shown to bind the same or overlapping epitopes, while two clones (A12 and 1:7) bound to one or two other epitopes on E2. None of the antibody clones recognized denatured E2 in a Western blot assay, strongly indicating that they recognize conformation-dependent epitopes. Such antibodies may be useful for assessing the conformational states of E2 during intracellular processing and virus assembly (Deleersnyder et al., 1997). Indeed, we noted higher ELISA reactivities for the antibodies to the E1/E2 complex compared to E2 with the same sequence. The E1/E2 complex is thought to contain the native form of E2 assembled on the virion, but only a few monoclonal antibodies distinguish well-folded E2 in heterodimers from other forms of E2 (Deleersnyder et al., 1997). To assess whether our antibodies may be used in this regard requires further studies. It is possible that differences in concentration of antigen account for the observed differences of reactivity.

Virus neutralizing antibodies are of particular interest for developing immunotherapy and as underlying bases for vaccine design (Chanock et al., 1993). In the case of HCV, assessing the possible neutralizing capacity of antibodies is difficult in the absence of a reliable in vitro neutralization assay. The NOB assay, measuring antibody inhibition of recombinant E2 binding to CD81 on target cells, may have relevance for neutralization although the extent to which CD81 is critical for the cell–HCV interaction has not been fully elucidated. Still, available data suggest that antibodies blocking this interaction may be protective in vivo (Ishii et al., 1998; Rosa et al., 1996). Four of our clones, representing specificities with distinct epitopes, were tested as purified, eukaryotic-expressed IgG1 for NOB activity: they were positive at concentrations of 0.3–0.5 µg/ml. Crude preparations of Fab molecules produced in E. coli showed higher NOB titres (0.001–2.5 µg/ml); the negative control Fab preparations remained negative. This discrepancy between NOB activity for the same clone if produced as Fab in bacteria or as IgG in CHO cells may have several explanations. First of all, the Fab preparations contain large amounts of bacterial substances, some of which may well have affinity for Ig-related structures. Second, Fab molecules expressed in bacteria have a tendency to aggregate. Both these factors may bias the ELISA used for quantification of Fab, and/or the cell-based NOB assay (including the secondary antibody reagents used in the latter). This problem is usually addressed by purifying the bacterial Fab. In the present study we instead moved the antibody genes to a different vector for expression in another host before purification of whole IgG. For some clones, the expression of eukaryotic genes in a eukaryotic host has been shown to provide antibody molecules of better efficiency, and is therefore to us more attractive (Samuelsson et al., 1996). In addition, it rules out the possibility that any remaining bacterial contaminants may produce artefactual results. Accordingly, we consider that data obtained with eukaryotic cell-produced IgG are more reliable for calculation of NOB activity, and conclude that the clones isolated are positive for blocking the E2–CD81 interaction at an approximate concentration of 0.5 µg/ml, a NOB titre in between those previously published for human anti-E2 antibodies (Fab and IgG) (Burioni et al., 1998; Habersetzer et al., 1998). We must also conclude that the NOB assay is not suitable for titrating crude periplasmic preparations of bacterially expressed Fabs. However, since the anti-HIV control Fab remained negative, the assay probably discriminates E2-binding Fabs from non-binding Fabs. Thus, all anti-E2 Fabs tested may actually bind E2 of both genotypes 1a and 1b, although some of the clones bound poorly to E2 genotype 1b coated to the solid phase in ELISA. This may suggest that the antibodies recognize only the small proportion of biologically active, well-folded E2 molecules – those active in CD81 binding (Flint et al., 2000).

Thus, the antibody clones were reactive with E2 of genotype 1a and 1b. The antibodies were very likely elicited by HCV type 2b (there was no E2 of genotype 2 available to us for testing the binding to this particular genotype). Taken together, these data suggest the existence of two or three cross-genotype conserved epitopes in HCV E2 that are conformation dependent, and are targets of antibody-mediated inhibition of the E2–CD81 interaction; as discussed above, such antibodies may be protective in vivo (Ishii et al., 1998; Rosa et al., 1996).

The use of monoclonal antibodies may help to better resolve the issue of protective antibodies to HCV. It is likely that natural infection induces only low titres of neutralizing antibodies, and may even result in mostly non-neutralizing interfering antibodies, as has been reported for HIV (Parren et al., 1997). Thus, serum from infected individuals may not be a good reagent for assessing protective antibodies to HCV, and the confusing results obtained so far must be interpreted with this in mind (Farci et al., 1992, 1994; Krawczynski et al., 1996). The present study represents one approach to overcome this problem.

In conclusion, we have isolated NOB-positive human monoclonal antibodies to HCV E2 that are reactive to two or three different conformational epitopes that seem to be conserved between different genotypes. Further assessment of
their antiviral properties has to await a reliable in vitro neutralization assay. Broadly reactive anti-E2 antibodies may be valuable for therapeutic applications and provide valuable knowledge for vaccine development.

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