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Anti-hepatitis C virus E2 (HCV/E2) glycoprotein monoclonal antibodies and neutralization interference

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ABSTRACT

The suggested HCV escape mechanism consisting in the elicitation of antibody (Ab) subpopulations interfering with the neutralizing activity of other Abs has recently been questioned. In particular, it was originally reported that Abs directed against the 436–447 region (epitope II) of HCV/E2 glycoprotein may interfere with the neutralizing Abs directed against the 412–423 region (epitope I) involved in the binding to CD81. In this paper, we investigate on the molecular features of this phenomenon describing an anti-HCV/E2 monoclonal Ab (mAb) (e509) endowed with a weak neutralizing activity, and whose epitope is centered on epitope II. Interestingly, e509 influenced the potent neutralizing activity of AP33, one of the best characterized anti-HCV/E2 mAb, whereas it did not show any interfering activity against two other broadly neutralizing mAbs (e20 and e137), whose epitopes partially overlap with that of e509 and which possibly displace it from the antigen.

These data may give a possible clue to interpret the conflicting studies published to date on the mechanism of interference, suggesting the existence of at least two groups of broadly neutralizing anti-HCV/E2 Abs: (i) those whose epitope is focused on the 412–423 CD81-binding region and whose activity may be hampered by other Abs directed against the 436–447 region, and (ii) those directed against CD81-binding regions but whose epitope contains also residues within the 436–447 region recognized by interfering mAbs, thus competing with them for binding. The conflicting results of previous studies may therefore depend on the relative amount of each of these two populations in the polyclonal preparations used. Overall, a better comprehension of this phenomenon may be of importance in the set up of novel mAb-based anti-HCV therapeutic strategies.

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, hepatocellular carcinoma, and liver cirrhosis (Alter et al., 1992; Makris et al., 1996). The development of an effective prophylactic and therapeutic approach against this virus has been hindered by its capability to mutate and to give rise to highly diversified viral variants (“quasispecies”) even within a single patient (Martell et al., 1992). Seven major genotypes varying by up to 30% in nucleotide sequence and several subtypes are recognized, each characterized by different clinical features, such as different evolutionary rates to chronic liver diseases or different response to available interferon-based antiviral therapies (Gottwein et al., 2009; Simmonds et al., 2005). HCV is actually the most common indication for liver transplantation (Armstrong et al., 2006). Post-transplant liver reinfection always occurs and its treatment is more problematic than in the pre-transplant setting, making the need of novel prophylactic and therapeutic approaches even more compelling (Brown, 2005).

The use of monoclonal antibodies (mAbs) directed against the surface E2 glycoprotein of HCV (HCV/E2) and capable of targeting structurally and functionally conserved regions on the highly variable viral particles is considered as a possible novel therapeutic strategy (Angus and Patel, 2011; Burioni et al., 2008). In fact, the production of potent neutralizing antibodies (Abs) in acute infections has been shown to correlate with viral clearance in a single-source outbreak cohort (Pestka et al., 2007). In vaccinated chimpanzees, a sustained Ab response to envelope glycoproteins E1 and E2 correlated with reduced viral load (Farci et al., 1994), while the passive administration of neutralizing mAbs in a uPA-SCID chimeric mouse model of infection was able to protect against challenge with a HCV quasispecies inoculum (Law et al., 2008).

Abbreviations: HCV, hepatitis C virus; HCV/E2, HCV E2 glycoprotein; aa, aminoacid; Ab, antibody; mAb, monoclonal antibody.

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Broadly cross-neutralizing anti-HCV/E2 human mAbs are typically directed against functionally important residues highly conserved among different genotypes (Broering et al., 2009; Johansson et al., 2007; Keck et al., 2008, 2004; Mancini et al., 2009; Owsianka et al., 2008; Perotti et al., 2008; Sabo et al., 2011). This aspect is crucial for the possible therapeutic *in vivo* use of such mAbs, but it may not be sufficient since it has been recently supposed that other Ab populations may interfere with their neutralizing activity (Burioni et al., 2001; Di Lorenzo et al., 2011; Zhang et al., 2009). In particular, studies with polyclonal human immunoglobulins have demonstrated that Abs recognizing the region encompassing residues 436–447 on HCV/E2 (also called epitope II) could impair the activity of neutralizing Abs directed against the 412–426 region (epitope I), a functionally important region involved in HCV binding to CD81 receptor (Zhang et al., 2009). Conversely, a recent study using murine mAbs and affinity-purified human immunoglobulins failed to demonstrate any inhibition between the two groups of Abs directed against the epitopes described above (Tarr et al., 2011).

As evident, none of the above studies investigated this phenomenon using combinations of human mAbs with different neutralizing features, and evaluating the resulting activity. In this work we investigated the interfering activity of a weakly neutralizing human anti-HCV/E2 mAb (e509), whose epitope is centered on epitope II, on the potent neutralizing features of two other human mAbs derived from the same patient (e137 and e20) (Bugli et al., 2001; Burioni et al., 2002; Mancini et al., 2009; Perotti et al., 2008), as well as on the well characterized broadly neutralizing mouse mAb AP33 (Owsianka et al., 2005).

2. Materials and methods

2.1. Cell culture, monoclonal antibodies and reagents

HEK-293T, HuH-7 (ATCC) and HuH-7.5 (Apath) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% inactivated foetal bovine serum (FBS, Euroclone), non-essential aminoacids and 50 mg/mL of penicillin and 100 mg/mL of streptomycin. Medium and all supplements, except FBS, were purchased from GIBCO (Life Technologies, Carlsbad, CA, USA).

The isolation of e20, e137 and e509 anti-HCV/E2 human mAbs has been previously described by Burioni et al. (1998); Bugli et al. (2001); Burioni et al. (2002); Mancini et al. (2009); Perotti et al. (2008). Together with e20 and e137, e509 is an anti-HCV/E2 human mAb cloned by phage display from the IgG1 light-chain κ repertoire of a genotype 1b HCV chronically infected patient with associated mixed cryoglobulinemia. Similarly to e20 and e137 (Mancini et al., 2009; Perotti et al., 2008), e509 is a conformation-sensitive Ab, as suggested by its inability to detect both denatured E2 by Western blot analysis and to bind, in ELISA, 15-mer peptide fragments with a 5-aminoacid N-terminal overlap covering the aminoacid sequence of the HCV/E2 of genotype 1a used (Burioni et al., 1998).

All the experiments described in this paper were conducted using the whole IgG1 format of the mAbs. To obtain the whole IgG, the BD BaculoGold System (BD Biosciences Pharmingen, San Diego, CA, USA) was used. Briefly, nucleotide sequences codifying heavy and light chains of e20, e137 and e509 Fab fragments were sub-cloned into the baculovirus expression vector pAc-k-Fc (PROGEN Biotechnik GmbH, Heidelberg, Germany). Sf-9 insect cells (Life Technologies, Carlsbad, CA, USA) were co-transfected with the linearized baculovirus DNA (BD Biosciences Pharmingen, San Diego, CA, USA) and the pAc-k-Fc/e20 or pAc-k-Fc/e137 or pAc-k-Fc/e509. The obtained viruses were employed at a multiplicity of infection (MOI) of 5 to infect 16109 H5 insect cells (Life Technologies, Carlsbad, CA, USA) in a final volume of 1 L. After an incubation of 96 h the culture media were collected, clarified by centrifugation and filtered with 0.2 μm filter (Millipore, Billerica, MA, USA). The media were loaded into a protein G column (Amersham Biosciences GE Healthcare, Zurich, Switzerland), the mAbs eluted with citric acid 0.1 M, pH 3.0, and immediately neutralized with Tris Base 1 M, pH 9.0. The solutions containing the mAbs were dialyzed against PBS and then concentrated using Amicon Ultra-15 Centrifugal Filter Devices (Millipore, Billerica, MA, USA). Ab concentration and purity (⩾95%) was determined by SDS–PAGE and by spectrophotometric measurement at 280 nm.

AP33 and ALP98 mouse mAbs were generously provided by A.H. Patel (MRC, Centre for Virus Research, University of Glasgow, UK) (Clayton et al., 2002) and H60 mouse mAb was a generous gift of J. Dubuisson (Inserm U1019, CNRS UMR8204, Center for Infection and Immunity of Lille, Université Lille Nord de France, France) (Flint et al., 1999).

2.2. Neutralization assays on HCV pseudoparticles (HCVpp) and on HCV cell culture (HCVcc) infectious viral particles

The neutralizing activity of AP33, e20, e137 and e509 mAbs used singularly or in a dual-reciprocal combination was tested using both a HCV pseudoparticle (HCVpp)-based and a cell culture infectious HCV (HCVcc)-based neutralization assays, following already described protocols (Mancini et al., 2009; Perotti et al., 2008; Wakita et al., 2005). For the HCVpp neutralization assays, prior to inoculation onto HuH-7 cells, HCVpp derived from murine leukemia virus (MLV) displaying unmodified and functional full-length E1–E2 proteins of genotype 1a (H77 strain), of genotype 1b (UKN1B5.23 strain) or of genotype 2a (JFH-1 strain) were generated, and titered by detection of a luciferase reporter gene activity, as previously described by Mancini et al. (2009); Perotti et al. (2008). In particular, HCVpp of all tested genotypes were reciprocally normalized using relative luminescence units (RLU). For the neutralization assay, a HCVpp infectious dose of approximately 10,000 RLU equivalents was incubated for 1 h at 37 °C with different concentrations of AP33, e20, e137 or e509, or of the dual-reciprocal combination of them in a equimolar ratio, or using e509 at a fixed saturating concentration (10 μg/mL), and mixed with the other mAbs at different concentrations. Three days following infection, the cells were analyzed on a luminometer (Victor3, Perkin Elmer) using the Bright-Glo™ luciferase assay kit (Promega) following the instructions provided by the manufacturer.

For the HCVcc neutralization assays, plasmids containing JFH-1 (genotype 2a) or H77/JFH-1 (a chimeric 1a/2a genome allowing the surface expression on HCVcc of genotype 1a envelope glycoproteins) genome cDNAs were used to produce HCVcc, as previously described by Mancini et al. (2009); Perotti et al. (2008); Pietschmann et al. (2006); Wakita et al. (2005). Cell supernatant was harvested and filtered through a 0.45 μm nitrocellulose membrane (Millipore, Billerica, MA, USA). At day 3 post-inoculation in HuH-7 or HuH-7.5, following immunofluorescence staining using a mouse mAb (9E10 clone) directed against the HCV/NS5A non structural protein (Lindenbach et al., 2005), enumeration of the foci of infected cells was performed through an *IN Cell Analyzer 2000* (GE Healthcare) platform and infectious virus titers were determined and expressed in focus-forming units of virus per milliliter (FFU/mL). For the neutralization assays, the different concentrations and combinations of mAbs were incubated with 100 FFU of virus for 1 h at 37 °C. The infectivity in the presence or absence (positive control) of the different concentrations and combination of mAbs was then evaluated as described above. Neutralization was then calculated as the percent reduction in FFU of virus.

2.3. Analysis of the binding activity

The binding activity of e20 and e137 mAbs using recombinant HCV/E2 proteins derived from different genotypes was previously described (Mancini et al., 2009; Perotti et al., 2008). Similarly to e20 and e137, the binding activity of e509 mAb was assayed using the following isolates: genotype 1a (isolate H77), 1b (isolate UKN1B5.23) and 2a (isolate JFH-1). In brief, HEK-293T cells were transfected with 3 μg of pcDNA3.1 vector, an expression vector encoding surface glycoproteins of the above listed HCV isolates (Perotti et al., 2008). After centrifugation, fixation and permeabilization, the transfected cells were incubated with e509 (10 μg/mL). After further washings, the cells were incubated with a FITC-conjugated anti-human Fc-IgG mAb (Sigma–Aldrich; 1:100), and analyzed by fluorescent microscopy and by FACS (FACSCalibur, BD Biosciences). Untransfected cells were also included in each experiment as negative controls.

AP33 was used to analyze the transfection efficiency for each genotype. The percentage of AP33-incubated sorted cells featuring a higher fluorescence signal than untreated cells was comparable for all different genotypes (approximately 70% of 10,000 cells analyzed by FACS). The binding activity of e509 against the different genotypes was then expressed as percentage of the reactivity observed on genotype 1a (relative binding activity), measured by sorting for each genotype the number of cells featuring a higher fluorescence signal than cells without mAb. The fluorescence obtained using e509 on untransfected cells was subtracted as background.

2.4. Affinity determination and competition assays in ELISA

Methods and results of competition assays using e20, e137 and e509 combinations were previously reported (Bugli et al., 2001; Burioni et al., 1998), and here confirmed using the mAbs as full IgG. Affinity determination and competition assays between AP33, e20, e137 and e509 were performed in ELISA as previously described by Bugli et al. (2001); Mancini et al. (2009); Perotti et al. (2008); Raghava and Agrewala (1994). In brief, for affinity determination, after coating ELISA plates (Costar, Corning, NY) with recombinant HCV/E2 (genotype 1a, strain H77) (Lesniewski et al., 1995) and blocking with PBS/BSA-1% (bovine serum albumin; Sigma–Aldrich) for 1 h at 37 °C, 40 μL of serial dilutions of testing mAb was added to the wells and the mixture was incubated for 1 h at 37 °C. After this step, plates were washed five times with PBS/Tween20–0.1% (Sigma–Aldrich), and binding of the mAb to the antigen was revealed with peroxidase-conjugated anti-mouse immunoglobulin serum (Sigma–Aldrich; 1:3,000) for AP33 or peroxidase-conjugated anti-human immunoglobulin serum (Sigma–Aldrich; 1:4,000) for e20, e137 and e509. After a final wash, 40 μL of TMB substrate (Thermo Scientific) was added and the optical density at 450 nm (OD450) of the plates were read after 15 min at 37 °C in the dark. The concentration (expressed in nM) giving approximately 50% of the maximum optical density at OD450 was considered the rough estimate of the mAb K_D value as previously reported (Raghava and Agrewala, 1994).

For competition assays, ELISA plates (Costar, Corning, NY) were coated with recombinant HCV/E2 (genotype 1a, strain H77) and blocked with PBS/BSA-1% for 1 h at 37 °C; subsequently, 40 μL of serial dilutions of a purified preparation of a competing mAb was added to the wells and the mixture was incubated for 2 h at 37 °C. After this step, an appropriate amount of probe mAb was added directly to the wells to obtain a final concentration giving approximately 60% of the maximum OD450 in the ELISA and the mixture was incubated for additional 30 min. Plates were then washed five times with PBS/Tween20–0.1%, and binding of the probe mAb to the antigen was revealed with a peroxidase-conjugated anti-mouse immunoglobulin serum (Sigma–Aldrich; 1:3000) if the probe mAb was AP33 or peroxidase-conjugated anti-human immunoglobulin serum (Sigma–Aldrich; 1:4,000) if the probe mAb was e20, e137 or e509. After a final wash, 40 μL of TMB substrate (Thermo Scientific) was added and the OD450 of the plates were read after 15 min at 37 °C in the dark. A negative control sample containing an excess of a purified control human mAb directed against the influenza virus hemagglutinin (anti-HA) (Clementi et al., 2011) and corresponding to 0% inhibition was included.

2.5. Epitope characterization: binding experiments on point-mutated HCV/E2 clones and multiple peptides of HCV/E2

In order to identify the residues involved in the interaction of AP33, e20, e137 and e509 with the HCV/E2 glycoprotein, the reactivity of the mAbs was evaluated against a panel of alanine point-mutated H77-derived HCV/E1-E2 (genotype 1a) molecules (except for A439 residue that we mutated in glycine). The mutated residues were identified among those crucial for the CD81-binding site (Owsianka et al., 2006), already investigated for AP33, e20 and e137, and within the interfering epitope recently described by Zhang et al. (aa 436–447) (Zhang et al., 2009). Part of the mutant panel was kindly provided by Alexander W. Tarr (Queen's Medical Centre, University of Nottingham, UK) (Owsianka et al., 2006). Other mutants were generated in this study by *Gene Taylor Mutagenesis kit* (Life Technologies, Carlsbad, CA, USA) using specific oligonucleotide primers designed following the instruction provided by the manufacturer.

Relative binding activity to mutated HCV/E1-E2 molecules, compared to the one against wild type, was determined in FACS experiments performed as described above. In this approach, the ALP98 mouse mAb (directed against a linear epitope outside the mutated regions) (Clayton et al., 2002) was used to evaluate the expression level of the mutated HCV/E1-E2 while a conformation-sensitive mouse mAb (H60) was used as folding control (Flint et al., 1999).

2.6. Statistical analysis

Neutralization data were analyzed as variable slope dose–response curves using Graphpad Prism version 5 sofware, and IC50 values determined using non-linear regression analysis.

Differences between means of neutralizing activities expressed as IC50 of mAbs used singularly and in combination were assessed using the Fisher's exact test, considering them as categorical variables (i.e. presence of interference when IC50 was influenced as in the case of e509 and AP33; lack of interference when IC50 was not altered).

3. Results

3.1. Evaluation of interfering activity in HCV neutralization assays

We investigated whether the weakly neutralizing e509 mAb was able to interfere with the potent neutralizing activity of AP33, whose binding region is within epitope I (aa 412–423) (Bugli et al., 2001; Owsianka et al., 2005, 2006; Tarr et al., 2006).

First of all, HCV pseudoparticles (HCVpp) of genotype 1a (H77 strain) were incubated with different concentrations of AP33, of e509, or of the combination of AP33 with e509 in an equimolar ratio. The results showed that AP33 neutralizing activity was negatively influenced by the presence of e509, determining a threefold increase of its IC50 (from 0.6 to 1.8 μg/mL) (Fig. 1A). A comparable IC50 increase was observed using AP33 at different concentrations

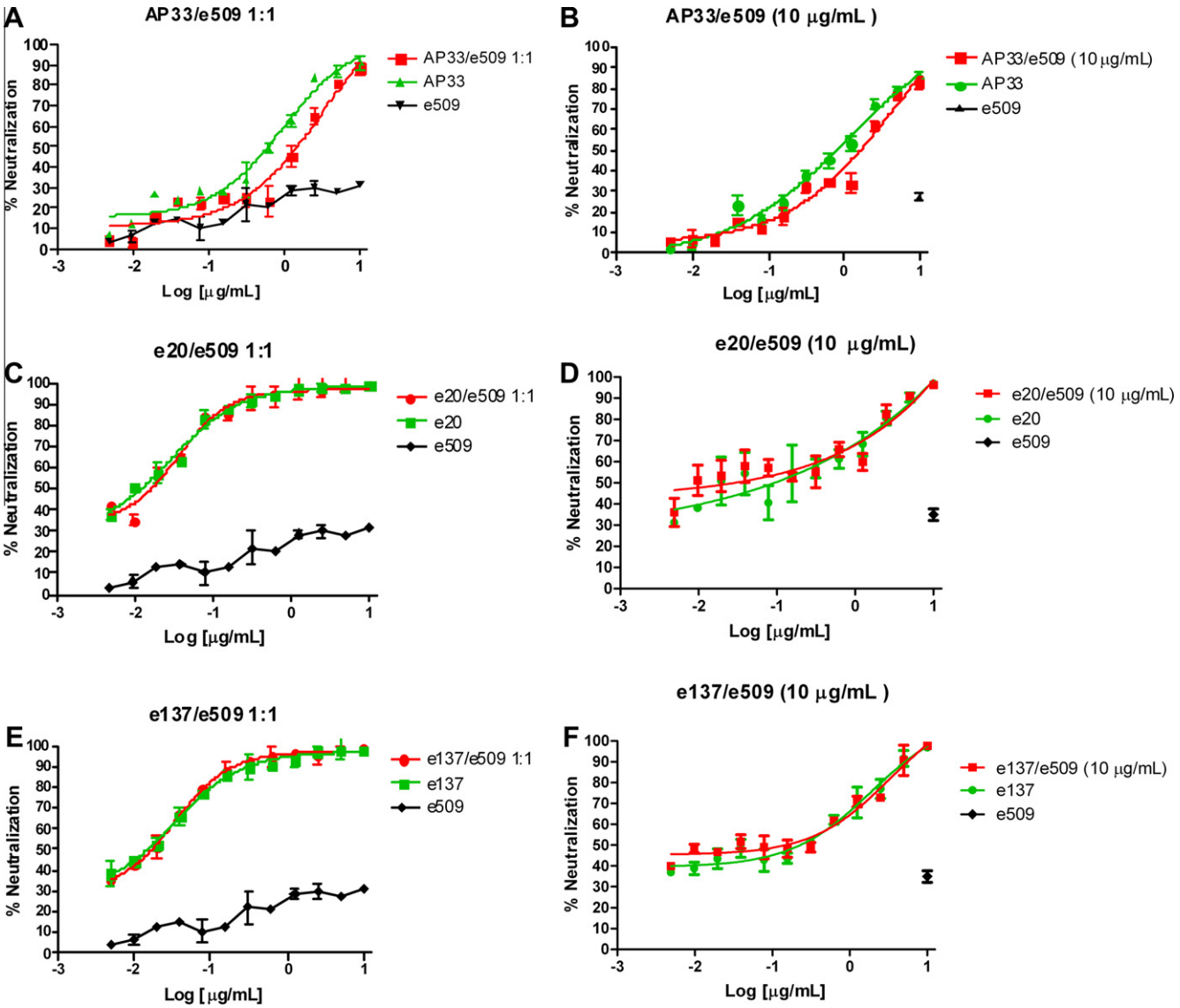

Fig. 1. Neutralization of HCVpp of genotype 1a using AP33, e20, e137 and e509 combinations. AP33, e20 and e137 mAbs (A, C and E, respectively) were used alone and in a 1:1 M ratio combination with e509 mAb or using increasing concentrations of AP33, e20 and e137 mAbs alone and in combination with e509 mAb at a fixed saturating concentration (10 μg/mL) (B, D and F, respectively). The concentration of each mAb, alone or in combination, is reported on *x* axis. The neutralizing activity (*y* axis) is expressed as percent reduction infectivity of HCVpp of genotype 1a (H77 strain) without mAbs. The experiment was performed three times in duplicate, and the means and standard errors (error bars) are reported.

in combination with e509 at a fixed saturating concentration (10 μg/mL) (Fig. 1B). Notwithstanding the higher intrinsic variability of the system, already reported by others (Tarr et al., 2011), a comparable trend of interference on AP33 neutralizing activity by e509 was also evidenced performing the same experiments using the cell culture (HCVcc) infectious chimera H77/JFH-1 (Supplementary Fig. 1A and B).

The above experiments were also performed using e509 in combination with two broadly neutralizing mAbs (e20 and e137) cloned from the same patient (Mancini et al., 2009; Perotti et al., 2008) but, interestingly, their neutralizing activity was not influenced by the presence of e509, showing unmodified IC50 values of 0.02 and 0.03 μg/mL, respectively (Fig. 1C–F). This observation was confirmed also using the HCVcc infectious chimera H77/JFH-1 (Supplementary Fig. 1C–F) The divergent effects observed on the IC50 of the three mAbs combined with e509 were corroborated using the Fisher's exact test ($p < 0.001$), showing the association between e509 and the slight interference on AP33 neutralizing activity.

The interfering role of e509 was also evaluated on HCVpp of genotype 1b (UKN1B5.23 strain) and of genotype 2a (JFH-1 strain), and on the authentic cell culture infectious clone (HCVcc) of genotype 2a (JFH-1 strain), but no interference was observed (Supplementary Fig. 2). This discrepancy could be only partially explained by the weaker binding of e509 to these genotypes (Fig. 2), although future studies better defining the residues involved in the binding to non-1a genotypes, and their spatial position on the protein, could possibly help in elucidating this aspect.

Moreover, unmodified neutralizing activities were observed when using the three neutralizing mAbs of the study (AP33, e20 and e137) in reciprocal combination against the HCVpp of genotype 1a (H77 strain) (Supplementary Fig. 3).

3.2. Affinity determination and competition assay in ELISA

In order to investigate the mechanisms leading to the slight e509 interference on AP33 neutralizing activity and its lack of interference on e20 and e137, we evaluated the relative affinity

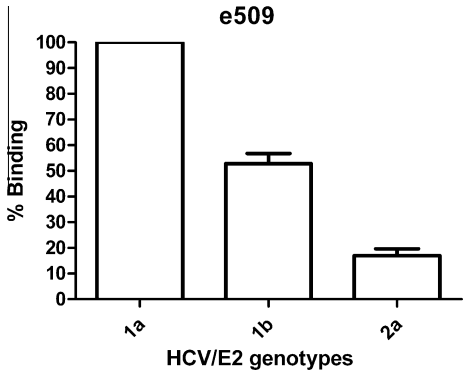

Fig. 2. Binding activity of e509 to HCV/E2 of different genotypes. HCV/E2 from genotype 1a (H77 strain); 1b (UKN1B5.23 strain) and 2a (JFH-1 strain). Binding activity for each genotype is expressed as percentage of the reactivity observed on HCV/E2 of genotype 1a (H77 strain). The means plus standard errors (error bars) for three replicate assays are reported. MAb e509 was tested at 10 μg/mL.

Table 1
Reaction pattern matrix showing competition of pairs of mAbs to HCV/E2 glycoprotein in ELISA. red, >60% inhibition; yellow, 25–60% inhibition; green, <25% inhibition. In the competition assays, a symmetrical behavior was observed for all combinations, but for AP33/e509 (*). Relative affinity for HCV/E2 (genotype 1a, strain H77) glycoprotein is enclosed in parentheses and determined in ELISA, as previously described by Raghava and Agrewala (1994). Competition ELISA experiments were performed as previously described by Bugli et al. (2001); Mancini et al. (2009); Perotti et al. (2008) and raw data are reported in Supplementary Fig. 4.

Competitor mAb	Probe mAb: e20	e137	e509	AP33
e20 (2 nM)				
e137 (0.4 nM)				
e509 (4.3 nM)				*
AP33 (9 nM)			*	

of each mAb for the antigen in ELISA (Matthews et al., 2011; Raghava and Agrewala, 1994). Although the Ab K_D measurement in ELISA provides only a rough estimate of the affinity of a mAb, it is however accepted in comparative studies (Raghava and Agrewala, 1994). Using this method AP33, e20 and e509 featured similar affinity values of 9, 2 and 4.3 nM, respectively, while e137 featured the highest affinity (0.4 nM) (Table 1).

Moreover, we evaluated the ability of e509 to inhibit the binding of AP33, e20 and e137 to HCV/E2 1a in a competitive ELISA (Bugli et al., 2001; Mancini et al., 2009; Perotti et al., 2008). The competition assays showed that all mAb combinations, with the exception of the AP33/e509 combination, featured a symmetrical behavior with e137 showing a high competition with AP33 and e20 and a moderate one with e509, similarly to the e20/e509 combination (Table 1 and Supplementary Fig. 4A–D). An intriguing aspect was the asymmetric competition observed between AP33 and e509, that is the stronger ability of e509 to interfere with AP33 binding compared to that of AP33 of inhibiting e509 (Table 1 and Supplementary Fig. 4A and D). These data give a possible clue to the interfering activity exerted by e509 on AP33 neutralizing activity, suggesting that their epitopes are contiguous but not overlapping and, in particular, that AP33 epitope could be partially masked or conformationally modified when e509 is already bound to E2 glycoprotein.

3.3. HCV/E2 alanine scanning

To better define the critical E2 residues involved, the binding of AP33, e20, e137 and e509 to a panel of alanine point-mutated H77-derived HCV/E1–E2 (1a) clones was determined. In particular, an alanine scanning mutagenesis was performed in the 436–447 region (epitope II), the interfering epitope described by Zhang et al. (2009). Moreover, we also evaluated the e509 binding features on two additional regions, 412–423 (epitope I) and 483–549, both critical for HCV/E2 binding to CD81 and already investigated for e20, e137 and AP33 (Mancini et al., 2009; Owsianka et al., 2006; Perotti et al., 2008; Tarr et al., 2006).

Importantly, the binding of e509 was totally abrogated by G436A, W437A, L438A, G440A, L441A, F442A and Y443A mutants and to a lesser extent by D535A mutant (Fig. 3), thus suggesting that its epitope is mainly centered on the interfering epitope II.

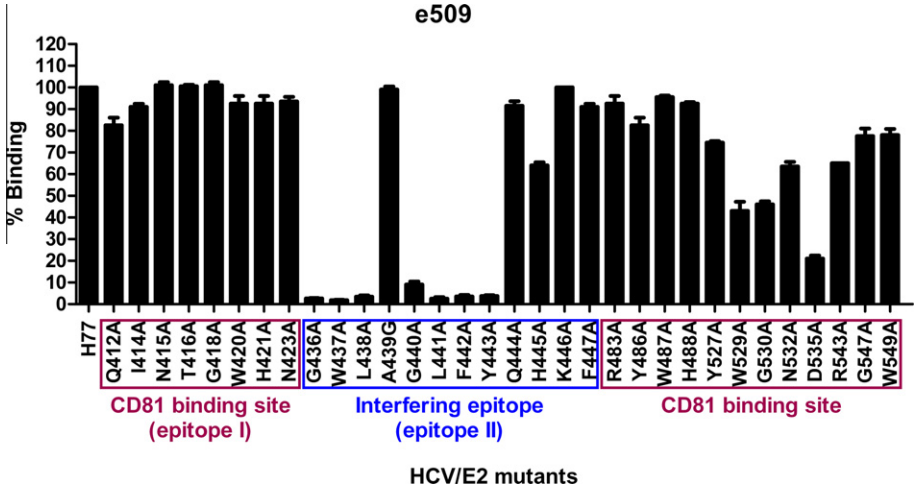

Fig. 3. Reactivity of e509 on point-mutated HCV/E2 glycoprotein (H77 strain). Mutations were generated within the interfering epitope (epitope II) discussed in the text (encompassing residues 436–447) and within two regions involved in HCV binding to CD81 (epitope I, encompassing residues 412–423, and the region involving residues 483–549). Binding experiments were performed as previously described, using a conformation-dependent non-competing anti-HCV/E2 mAb (H60) (Supplementary Fig. 5) to check for correct protein folding of each mutant and ALP98 mAb to check for E2 glycoprotein expression levels (Mancini et al., 2009; Owsianka et al., 2006; Perotti et al., 2008; Tarr et al., 2006). Binding activity is expressed as a percentage of the reactivity on wild-type HCV/E2 (H77 strain). The means plus standard errors (error bars) for three replicate assays are reported. MAb e509 was tested at 10 μg/mL.

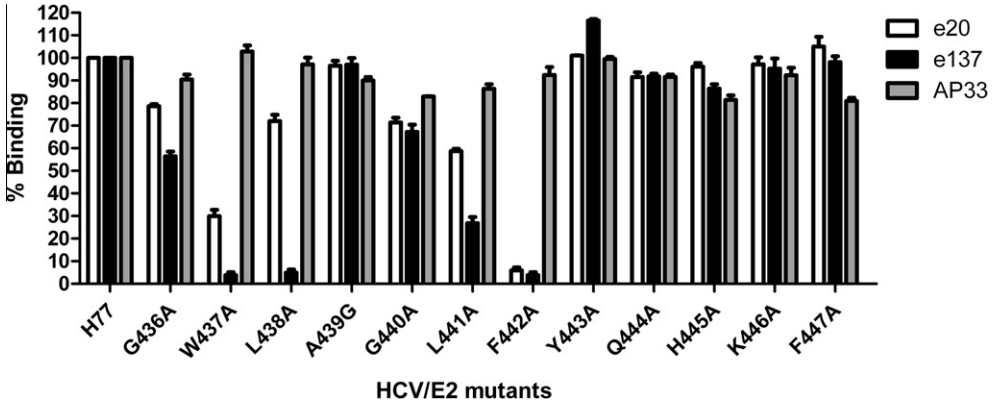

Fig. 4. Reactivity of e20, e137 and AP33 on point-mutated HCV/E2 glycoprotein (H77 strain). The mutated region involves residues within the interfering epitope II (residues 436–447). Binding experiments were performed as previously described, using a conformation-dependent non-competing anti-HCV/E2 mAb (H60) to check for correct protein folding of each mutant (Supplementary Fig. 5) and ALP98 mAb to check for E2 glycoprotein expression levels (Mancini et al., 2009; Owsianka et al., 2006; Perotti et al., 2008; Tarr et al., 2006). Binding activity is expressed as percentage of the reactivity on unmutated wild-type HCV/E2 (H77 strain). The means plus standard errors (error bars) for three replicate assays are reported. MAbs e20, e137 and AP33 were tested at 10 μg/mL.

On the other hand, AP33 (whose binding is known to be centered on epitope I) was not affected by any of the mutants within epitope II (Owsianka et al., 2005). Conversely, e20 binding was drastically reduced by W437A and F442A mutants (Fig. 4), suggesting that its epitope contains residues within the interfering epitope II. However, its neutralizing activity is not due to the binding of residues within epitope I but to other CD81-binding residues (W529, G530 and D535) outside it (Bugli et al., 2001; Mancini et al., 2009; Perotti et al., 2008). Finally, and more interestingly, the binding of e137, previously demonstrated to be reduced by T416A, W420A (epitope I) and W529A, G530A, D535A (within the other known CD81-binding region) (Owsianka et al., 2006; Perotti et al., 2008), was also decremented by W437A, L438A, L441A and F442A mutants within the interefering epitope II (Fig. 4).

It is noteworthy that none of the HCV/E2 mutants affect the overall glycoprotein conformation as demonstrated by comparable binding activity featured by the control mAb (H60) to the wild-type HCV/E2 glycoprotein (Supplementary Fig. 5).

4. Discussion

The possible role of a mechanism of Ab interference in HCV immune escape has recently been proposed and debated (Burioni et al., 2001; Di Lorenzo et al., 2011; Tarr et al., 2011; Zhang et al., 2009), but it has never been verified using human anti-HCV/E2 mAbs isolated from the same patient and used in reciprocal combinations. In this work, we report that a weakly neutralizing human mAb (e509) (Bugli et al., 2001), whose epitope is focused within the 436–447 aminoacid region (epitope II) of HCV/E2, is able to slightly interfere with the neutralizing activity of the mouse mAb AP33. AP33 is one of the broadest cross-neutralizing mAb described to date and it is directed against the linear epitope encompassed by residues 412–423 (epitope I) contiguous to the possible interfering epitope II (Owsianka et al., 2006; Tarr et al., 2006). The lack of common mutated residues able to decrease the binding of both e509 and AP33 and the asymmetric competition observed between the two mAbs in this paper suggest that the binding of e509 to the region containing epitope II may interfere with AP33 activity by sterically hindering its binding to epitope I, as originally suggested by Bugli et al. (2001); Burioni et al. (2002, 1998); Krey et al. (2010); Zhang et al. (2009). At this regard, another possibility is that the initial binding of e509 to the region containing epitope II may induce conformational changes on E2 that inhibit AP33 interaction with epitope I, as recently suggested by Lapierre et al. for other anti-HCV/E2 Abs (Lapierre et al., 2011).

Interestingly, an apparent reduction of the weak neutralizing activity of e509 is evident in the HCVpp assay when in combination with low concentrations of AP33 (Fig. 1B). This peculiar interfering effect of AP33 was detectable also in the HCVcc system, even if the higher variability made it less evident (Supplementary Fig. 1B). These data are probably another sign of the close interplay between the regions bound by the two mAbs. More importantly, a recent paper on a phase 1 safety and immunogenicity vaccinal trial with HCV envelope proteins evidenced a paradoxical HCVcc-enhancing effect of sera with a low titer of anti-HCV/E2 neutralizing antibodies (Meyer et al., 2011). As already reported for other viruses such as West Nile virus (Sullivan, 2001), the observed AP33 interfering effect could be another evidence that, also in the case of HCV, neutralizing Abs may have dramatically divergent biological activities when used at extremely different concentrations.

The mechanism of interference of Abs directed against epitope II was recently reported also on the neutralizing activity of Abs directed against the 517–531 epitope, the CD81 binding region recognized by the two other cross-neutralizing anti-HCV/E2 human mAbs (e20 and e137) used in this study (El Abd et al., 2011). We are not able to support such a finding since e20 and e137 are not apparently influenced by e509 (Mancini et al., 2009; Perotti et al., 2008). However, two observations emerging from this study may possibly contribute to the observed lack of interference: (i) both e20 and e137 mostly bind residues targeted by neutralizing Abs in a CD81-binding region outside epitope I and therefore potentially less subjected to the interfering effect of epitope II-directed Abs (Mancini et al., 2009; Perotti et al., 2008); and (ii) both mAbs (in particular e137) also bind residues within the interfering epitope II itself, but at higher affinity than e509 thus displacing it from HCV/E2.

Conversely, it is important to remember that another recent study by Tarr et al. failed to evidence any interfering activity exerted by Abs directed against epitope II (Tarr et al., 2011). As admitted by the authors themselves, these divergent data could derive from the different composition of each single polyclonal preparations used, each possibly enriched in clones with peculiar features. The diverging effects observed in our study using single mAbs strongly support this hypothesis. Another interesting aspect is that the three human mAbs (e20, e137 and e509), binding residues within epitope I or epitope II, came from the same patient. As previously speculated (Tarr et al., 2011; Zhang et al., 2009), this may be interpreted as a possible novel immune escape mechanisms of HCV, since Abs with such completely different biological

activity are really produced during an infection, and are directed against very close, almost overlapping, regions on HCV/E2. The high variability of the region encompassing epitope II, also called highly variable region 3 (HVR3), further complicates this scenario (Troesch et al., 2006). In other words, the infecting genotype/isolate may be another reason for not observing a possible interfering mechanism. In our study, this is somehow suggested by the lower binding of e509 on HCV/E2 of genotypes 1b and 2a, as well as by the lack of interference of e509 on AP33 against these genotypes. Another factor not investigated in this study, but possibly influencing the observed genotype-dependent effect, is the spatial position of the regions bound by our mAbs on the different genotypes. Indeed, as shown by our group using anti-influenza mAbs, the different biological effects of a mAb directed against a highly conserved region of an hypervariable virus may be related to the position of its bound region on different viral variants (Clementi et al., 2011; De Marco et al., 2012).

5. Conclusions

Considering our data and recent published works, it is possible to conclude that epitope II is the target of Abs with different neutralizing features (Drummer et al., 2006; Edwards et al., 2012; Keck et al., 2012; Lapierre et al., 2011; Zhang et al., 2009). Our data may be interpreted as a good compromise between the interfering nature of epitope II hypothesized by Zhang et al. and the absence of such interfering activity demonstrated by Tarr et al. that conversely also suggests that Abs recognizing epitope II contribute to the neutralizing activity of Abs recognizing epitope I (Tarr et al., 2011).

Beside being potentially useful for a better comprehension of the interplay between HCV and the humoral response, these observations may also be of practical importance. Our work strongly suggests that mAbs to be possibly used in anti-HCV passive immunization approaches, or to be elicited by future vaccine strategies, have not only to be highly cross-neutralizing molecules targeting key conserved residues, but also tailored molecules whose activity is not influenced by possible interfering Abs produced in some occasions. To this end, they must either be directed against conserved neutralizing epitopes not subjected to the mechanism of interference or, as in the case of e137, they must be able to prevent the binding of the interfering Abs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.07.013.

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