Neutralization activity and kinetics of two broad-range human monoclonal IgG1 derived from recombinant Fab fragments and directed against Hepatitis C Virus E2 glycoprotein

Roberta Antonia Diotti, Giuseppe Andrea Sautto, Laura Solforosi, Nicasio Mancini, Massimo Clementi, Roberto Burioni

Laboratory of Microbiology and Virology, Università Vita-Salute San Raffaele, Milano, Italy

SUMMARY

Hepatitis C virus (HCV) is the major cause of chronic liver disease worldwide. There is evidence that neutralizing anti-HCV antibodies may find potential applications in novel prophylactic and therapeutic strategies. This paper describes the very high neutralization activity and unique biological features of two broadly cross-reactive and cross-neutralizing anti-HCV human monoclonal IgG1 derived from human monoclonal recombinant Fab fragments.

KEY WORDS: Hepatits C virus (HCV), Neutralizing anti-HCV antibodies, E2 glycoprotein.

Received August 02, 2012 *Accepted* September 05, 2012

Hepatitis C virus (HCV) infects 170 million people worldwide and causes chronic liver inflammation and fibrogenesis leading to cirrhosis, end-stage liver failure and hepatocellular carcinoma (Alter, 1997; Seeff *et al.,* 1992; Tong *et al.,* 1995). HCV infection is also associated with several extra-hepatic complications, in particular to cryoglobulinemia (Perotti *et al.,* 2008a; Sautto *et al.,* 2012b). The development of an effective anti-HCV prophylactic or therapeutic strategy has been hindered by the ability of this virus to continuously mutate (Deutsch and Hadziyannis, 2008). The current standard treatment of HCV is based on a combination therapy (ribavirin and interferon) fraught with side-effects and associated with a low sustained virological response (50-55%), especially when used against the most common and "aggressive" HCV genotype (genotype 1) (Mira *et al.*, 2009). Some hope has been recently stimulated by the introduction of anti-HCV protease inhibitors (boceprevir, telaprevir), but several studies have already reported a low mutational barrier against these drugs, especially when used in monotherapy, with the rapid emergence of resistant quasispecies (Forestier *et al.,* 2007; Zhou *et al.,* 2008). Overall, notwithstanding recent advances, there is still a great need for novel alternative anti-HCV strategies.

Increasing evidence supports the beneficial role of neutralizing antibodies in different viral infections (Burioni *et al.,* 2010; Burioni *et al.,* 2008; Clementi *et al.,* 2011;), including HCV (Chandra *et al.,* 2010; Pestka *et al.,* 2007; Yu *et al.,* 2004). On these basis, the availability of anti-HCV monoclonal antibodies capable of targeting structurally and functionally conserved regions on the highly variable surface viral proteins is of extreme importance (Burioni *et al.,* 2008; Johansson *et al.*, 2007). In our laboratory, we previously isolated and characterized two broadly cross-neutralizing anti-HCV E2 human monoclonal Fab fragments named Fab-e20 and Fab-e137 (Mancini *et al.,* 2009; Perotti *et al.,* 2008b). These recombinant Fab fragments were obtained against HCV\E2 (Burioni *et al.,* 1998b) using phage display-based

Corresponding author
Roberta Antonia Diotti, Ph.D.
Laboratorio di Microbiologia e Virologia
Università Vita-Salute San Raffaele
Via Olgettina, 58 - 20132 Milano, Italy
E-mail: diotti.robertaantonia@hsr.it

strategies, a technique for cloning and optimization of monoclonal antibodies directed against human pathogens (Clementi *et al.,* 2012; Mancini *et al.,* 2004; Solforosi *et al.,* 2012). However, the Fab fragment is characterized by a shorter half-life and is not endowed with many of the biological activities of the whole IgG1, that are often needed for a full exploitation of the clinical potential of the molecule (Jiang *et al.*, 2011). In addition, the non-Fc-related biological activity of a Fab molecule often increases substantially when using it as a whole IgG (Clementi *et al.,* 2011; De Marco *et al.,* 2012). For this reason, the genes coding for the Fab molecules were used to produce the corresponding IgG1 molecules. In this paper, we describe the biological activity, the binding characteristics and the neutralization kinetics of these two novel anti-HCV human monoclonal IgG1.

The whole IgG1 antibodies IgG1-e20 and IgG1-e137 were generated from the corresponding Fab-e20 and Fab-e137 using the BD BaculoGold System (BD Biosciences Pharmingen), and purified as described (Sautto *et al.,* 2012a) to a purity ≥95%. Reference Fabs were produced and purified as described (Burioni *et al.,* 1998a). When tested in ELISA (Lesniewski *et al.,* 1995), the two IgG1 molecules strongly reacted against HCV/E2 showing a 10-fold-higher affinity (ranging from 1 to 5 nM) than the corresponding Fab fragments against recombinant HCV/E2 protein derived from genotype 1a (strain H77) (data not shown). For a better definition of the cross-reactivity featured by the two antibodies, IgG1-e20 and IgG1-e137 were tested in immunofluorescence assay against human epithelial kidney (HEK) 293T cells expressing HCV E1-E2 from all six HCV genotypes (1a, 1b, 2a, 2b, 3, 4, 5, 6). Both IgG1 antibodies showed the capacity of binding all HCV genotypes tested, evidencing the highly conserved nature of their epitopes (data not shown). Remarkably, while Fab-e137 did not react against cells expressing genotype 5 glycoprotein, the corresponding IgG1-e137 acquired the ability to recognize also this genotype. This discrepancy can be due to the above mentioned fact that it is well known that human monoclonal Fabs directed against variable viral targets, once expressed in their "original" molecular structure, often acquire a wider reactivity (Clementi *et al.,* 2011).

Fab-e20 and Fab-e137 epitopes were described as including conserved residues that are crucial for CD81 binding (Mancini *et al.,* 2009; Perotti *et al.,* 2008b), and it was important to demonstrate that this desirable feature was retained by the corresponding IgG1 molecules. As expected, IgG1-e20 and IgG1-e137 maintained the ability of in-

TABLE 1 - *Neutralizing activity of e20, e137 IgGs and Fabs using virus pseudoparticles (HCVpp) and cell culture infection HCV (HCVcc). The table lists IgGs and Fabs concentrations giving 50% neutralization (IC50). Neutralizing activity experiments were performed using HCV pseudoparticles derived from murine leukemia virus (MLV) displaying unmodified and functional full-length E1-E2.*

Genotype	*IgG e20* IC_{50} *µg/mL*	*Fab e20* IC_{50} *µg/mL*	*IgG e137* IC_{50} *µg/mL*	*Fab e137* IC_{50} *µg/mL*
HCVpp 1a (H77)	0.02	7.5	0.03	5
HCVpp 1b (5.23)	0.3	>15	0.9	20
HCVpp 2a (1.2)	1.2	7.5	1.6	N.D.
HCVpp 2b (1.7)	0.3	>30	0.9	20
HCVpp 3a (4.28)	1	N.D.	1	N.D.
HCVpp 4 (21.6)	1	1.6	1.4	5
HCVpp 5 (16.11)	0.7	N.D.	1	N.D
HCVcc 2a (JFH1)	<0.3	2.5	<0.3	2.5

N.D. not determined.

terfering with the HCV/E2-CD81 binding. In particular at a concentration of 10 µg/mL the inhibition of binding was about 80% for both IgGs, higher than that observed with the corresponding Fabs (50%).

Obviously, the most important activity for an antiviral antibody is the capacity of blocking viral infectivity, and for this purpose IgG1-e20 and IgG1-e137 were comparatively tested against the corresponding Fabs against HCV pseudoparticles (HCVpp) derived from murine leukemia virus displaying unmodified and functional full-length E1-E2 proteins of genotypes 1 to 5 (Bartosch *et al.*, 2003). Both IgG1-e20 and IgG1-e137 showed a strong neutralization activity against all tested genotypes (Table 1), with a remarkable increase of potency when compared with the corresponding Fabs. In particular, the neutralizing activity of both IgGs on HCVpp/1a was extremely high (Table 1), and IgG1-e20 and IgG1-e137 resulted to be the most powerful neutralizing anti-HCV monoclonal antibodies described to date.

Furthermore, the neutralizing activity of IgG1-e20 and IgG1-e137 was also evaluated using the cell culture infectious HCV (HCVcc) system based on HCV genotype 2a strain JFH-1 (Wakita *et al.*, 2005). As expected from the HCVpp data, both antibodies showed a strong neutralizing activity also in this replicative HCV model (Table 1).

Antibody neutralization may involve different stages of viral infection, including attachment, internalization, or fusion. To identify the stage of viral cycle at which IgG1-e20 and IgG1-e137 interfere with the viral cycle, we used a pre- and post-attachment inhibition assay originally developed by Haberstroh *et al.* (Haberstroh *et al.*, 2008). In this assay, the antibodies are incubated with HCVpp of genotype 1a before or after the binding to Huh-7.5 cells at 4°C, and the level of infection is then measured. IgG1-e20 and IgG1-e137 showed a pattern of inhibition similar to the one observed for antibodies directed against the viral cellular receptors CD81, confirming that the targeted entry step is closely linked to HCV-CD81 interaction (Figure 1A). To further investigate this aspect, we comparatively evaluated the inhibitory capacity of IgG1-e20, IgG1-e137, anti-CD81 antibody and heparin in a kinetic study in which, following protocol II, the inhibitory agents were added at various time points post-binding. This assay showed that IgG1-e20 and IgG1-e137 lost potency in blocking entry when added at 30 minutes post-attachment, suggesting that they interfere with the early post-binding steps of viral entry (Figure 1B).

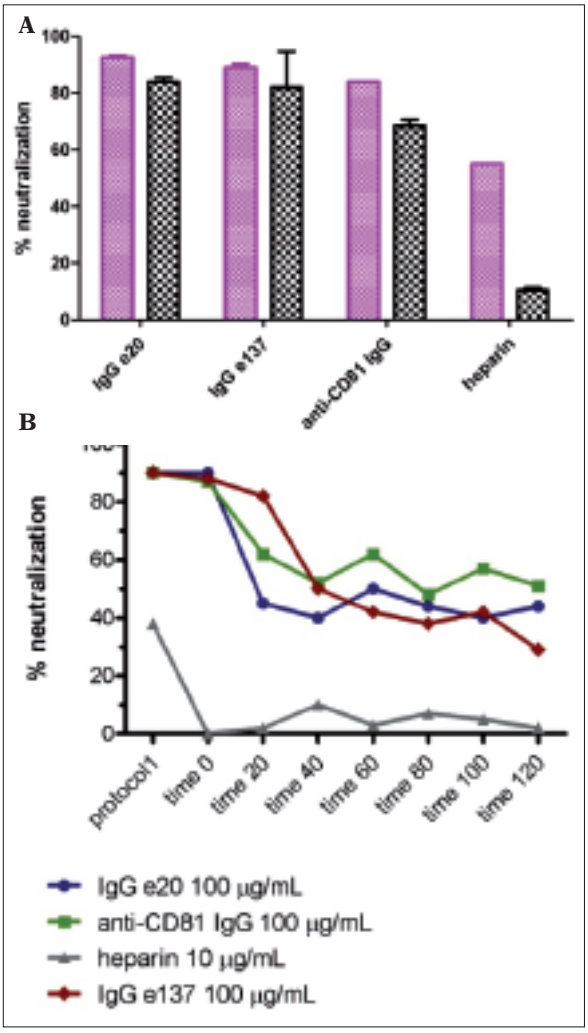

FIGURE 1 - *Neutralizing responses in HCV infection target HCVpp entry at post-binding step. (A) inhibition of HCVpp (1a, H77 strain) entry into HuH-7.5 cells by anti-CD81 antibody, IgG e20, IgG e137 and heparin in protocol I and II (In protocol I, violet, inhibitory antibodies were added before binding of HCVpp to the target cells. In protocol II, black, antibodies were added after HCVpp binding to target cells). (B) Kinetics of antibody-mediated inhibition of HCVpp at different time point (every 20 minutes, up to 120 minutes, after binding of HCVpp to HuH-7.5) by anti-CD81 antibody, IgG e20, IgG e137 and heparin. The inhibition kinetics and the comparison with inhibitors directed against known targets evidence that both antibodies block viral entry at an early post-binding step.*

In conclusion, we described two broadly cross-reactive and cross-neutralizing anti-HCV/E2 antibodies, IgG1-e20 and IgG1-e137. As the corresponding Fabs, IgG1-e20 and IgG1-e137 recognize a discontinuous epitope requiring residues within the CD81 binding site on E2 glycoprotein. Moreover, IgG1-e20 and IgG1-e137 showed similar kinetic of inhibition of HCV entry observed for anti-CD81 antibodies, indicating that they interfere at an early post-binding step. Moreover, an important issue in the role played by antibodies in the interplay between the host and HCV is neutralization interference by non-neutralizing antibodies (Burioni *et al.,* 2001; Tarr *et al.,* 2012; Zhang *et al.,* 2009). Importantly, the two IgG1 described here have been described as not being affected by interfering antibodies in their neutralizing activity (Sautto *et al.,* 2012a)
Our results demonstrate that IgG1-e20 and IgG1-e137 retain all desired features and have improved biological activity when compared to the corresponding Fabs. These data are encouraging in the quest for a potent cocktail of broad neutralizing monoclonal antibodies that could represent a novel anti-HCV therapy.

REFERENCES

Alter M.J. (1997). The epidemiology of acute and chronic hepatitis C. *Clin. Liver. Dis.* **1** (3), 559-568, vi-vii.

Bartosch B., Dubuisson J., Cosset F.L. (2003). Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J. Exp. Med.* **197** (5), 633-642.

Burioni R., Bugli F., Mancini N., Rosa D., Di Campli C., Moroncini G., Manzin A., Abrignani S., Varaldo P.E., Clementi M., Fadda G. (2001). Non neutralizing human antibody fragments against hepatitis C virus E2 glycoprotein modulate neutralization of binding activity of human recombinant Fabs. *Virology.* **288** (1), 29-35.

Burioni R., Canducci F., Mancini N., Clementi N., Sassi M., De Marco D., Diotti R.A., Saita D., Sampaolo M., Sautto G., Pianezze M., Clementi M. (2010). Monoclonal antibodies isolated from human B cells neutralize a broad range of H1 subtype influenza A viruses including swine-origin Influenza virus (S-OIV). *Virology.* **399** (1), 144-152.

Burioni R., Perotti M., Mancini N., Clementi M. (2008). Perspectives for the utilization of neutralizing human monoclonal antibodies as anti-HCV drugs. *J. Hepatol.* **49** (2), 299-300.

Burioni R., Plaisant P., Bugli F., Delli Carri V., Clementi M., Fadda G. (1998a). A vector for the expression of recombinant monoclonal Fab fragments in bacteria. *J. Immunol. Methods.* 217 (1-2), 195-199.

Burioni R., Plaisant P., Manzin A., Rosa D., Delli Carri V., Bugli F., Solforosi L., Abrignani S., Varaldo P.E., Fadda G., Clementi M. (1998b). Dissection of human humoral immune response against hepatitis C virus E2 glycoprotein by repertoire cloning and generation of recombinant Fab fragments. *Hepatology.* **28** (3), 810-814.

Chandra P.K., Hazari S., Poat B., Gunduz F., Prabhu R., Liu G., Burioni R., Clementi M., Garry R.F., Dash S. (2010). Intracytoplasmic stable expression of IgG1 antibody targeting NS3 helicase inhibits replication of highly efficient hepatitis C Virus 2a clone. *Virol. J.* **7**, 118.

Clementi N., De Marco D., Mancini N., Solforosi L., Moreno G.J., Gubareva L.V., Mishin V., Di Pietro A., Vicenzi E., Siccardi A.G., Clementi M., Burioni R. (2011). A human monoclonal antibody with neutralizing activity against highly divergent influenza subtypes. PLoS One. **6** (12), e28001.

Clementi N., Mancini N., Solforosi L., Castelli M., Clementi M., Burioni R. (2012). Phage display-based strategies for cloning and optimization of monoclonal antibodies directed against human pathogens. *Int. J. Mol. Sci.* **13** (7), 8273-8292.

De Marco D., Clementi N., Mancini N., Solforosi L., Moreno G.J., Sun X., Tumpey T.M., Gubareva L.V., Mishin V., Clementi M., Burioni R. (2012). A non-VH1-69 heterosubtypic neutralizing human monoclonal antibody protects mice against H1N1 and H5N1 viruses. PLoS One **7** (4), e34415.

Deutsch M., Hadziyannis S.J. (2008). Old and emerging therapies in chronic hepatitis C: an update. *J. Viral. Hepat.* 15 (1), 2-11.

Forestier N., Reesink H.W., Weegink C.J., McNair L., Kieffer T.L., Chu H.M., Purdy S., Jansen P.L., Zeuzem S. (2007). Antiviral activity of telaprevir (VX-950) and peginterferon alfa-2a in patients with hepatitis C. *Hepatology.* **46** (3), 640-648.

Haberstroh A., Schnober E.K., Zeisel M.B., Carolla P., Barth H., Blum H.E., Cosset F.L., Koutsoudakis G., Bartenschlager R., Union A., Depla E., Owsianka A., Patel A.H., Schuster C., Stoll-Keller F., Doffoel M., Dreux M., Baumert T.F. (2008). Neutralizing host responses in hepatitis C virus infection target viral entry at postbinding steps and membrane fusion. *Gastroenterology.* **135** (5), 1719-1728 e1.

Jiang X.R., Song A., Bergelson S., Arroll T., Parekh B., May K., Chung S., Strouse R., Mire-Sluis A., Schenerman M. (2011). Advances in the assessment and control of the effector functions of therapeutic antibodies. *Nat. Rev. Drug. Discov.* **10** (2), 101-111.

Johansson D. ., Voisset C., Tarr A.W., Aung M., Ball

J.K., Dubuisson J., Persson M.A. (2007). Human combinatorial libraries yield rare antibodies that broadly neutralize hepatitis C virus. *Proc. Natl. Acad. Sci USA*. **104** (41), 16269-16274.

Lesniewski R., Okasinski G., Carrick R., Van Sant C., Desai S., Johnson R., Scheffel J., Moore B., Mushahwar I. (1995). Antibody to hepatitis C virus second envelope (HCV-E2) glycoprotein: a new marker of HCV infection closely associated with viremia. *J. Med. Virol.* **45** (4), 415-422.

Mancini N., Carletti S., Perotti M., Canducci F., Mammarella M., Sampaolo M., Burioni R. (2004). Phage display for the production of human monoclonal antibodies against human pathogens. *New Microbiol.* **27** (4), 315-328.

Mancini N., Diotti R.A., Perotti M., Sautto G., Clementi N., Nitti G., Patel A.H., Ball J.K., Clementi M., Burioni R. (2009). Hepatitis C virus (HCV) infection may elicit neutralizing antibodies targeting epitopes conserved in all viral genotypes. PLoS One. **4** (12), e8254.

Mancini N., Solforosi L., Clementi N., De Marco D., Clementi M., Burioni R. (2011). A potential role for monoclonal antibodies in prophylactic and therapeutic treatment of influenza. *Antiviral. Res.* **92** (1), 15-26.

Mira J.A., Gutierrez-Valencia A., Gil Ide L., Merino D., Rivero A., Rios-Villegas M.J., Delgado M., Gonzalez-Serrano M., Collado A., Torres-Tortosa M., Omar M., Lopez-Ruz M.A., Macias J., Arponen S., Pineda J.A. (2009). Efficacy and safety of pegylated interferon plus ribavirin in HIV and hepatitis C virus-coinfected patients with advanced immunosuppression. *Clin. Infect. Dis.* **49** (8), e84-91.

Perotti M., Ghidoli N., Altara R., Diotti R.A., Clementi N., De Marco D., Sassi M., Clementi M., Burioni R., Mancini N. (2008a). Hepatitis C virus (HCV)-driven stimulation of subfamily-restricted natural IgM antibodies in mixed cryoglobulinemia. *Autoimmun. Rev.* **7** (6), 468-472.

Perotti M., Mancini N., Diotti R.A., Tarr A.W., Ball J.K., Owsianka A., Adair R., Patel A.H., Clementi M., and Burioni R. (2008b). Identification of a broadly cross-reacting and neutralizing human monoclonal antibody directed against the hepatitis C virus E2 protein. *J. Virol.* **82** (2), 1047-1052.

Pestka J.M., Zeisel M.B., Blaser E., Schurmann P., Bartosch B., Cosset F.L., Patel A.H., Meisel H., Baumert J., Viazov S., Rispeter K., Blum H.E., Roggendorf M., Baumert T.F. (2007). Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc. Natl. Acad. Sci. USA.* **104** (14), 6025-6030.

Sautto G., Mancini N., Diotti R.A., Solforosi L., Clementi M., Burioni R. (2012a). Anti-hepatitis C virus E2 (HCV/E2) glycoprotein monoclonal antibodies and neutralization interference. *Antiviral. Res.* **96** (1), 82-89.

Sautto G., Mancini N., Solforosi L., Diotti R.A., Clementi M., Burioni R. (2012b). HCV proteins and immunoglobulin variable gene (IgV) subfamilies in HCV-induced type II mixed cryoglobulinemia: a concurrent pathogenetic role. *Clin. Dev. Immunol.* **2012**, 705013.

Seeff L.B., Buskell-Bales Z., Wright E.C., Durako S.J., Alter H.J., Iber F.L., Hollinger F.B., Gitnick G., Knodell R.G., Perrillo R.P., et al. (1992). Long-term mortality after transfusion-associated non-A, non-B hepatitis. The National Heart, Lung, and Blood Institute Study Group. *N. Engl. J. Med.* **327** (27), 1906-1911.

Solforosi L., Mancini N., Canducci F., Clementi N., Sautto G.A., Diotti R.A., Clementi M., Burioni R. (2012). A phage display vector optimized for the generation of human antibody combinatorial libraries and the molecular cloning of monoclonal antibody fragments. *New Microbiol.* **35** (3), 289-294.

Tarr A.W., Urbanowicz R.A., Jayaraj D., Brown R.J., McKeating J.A., Irving W.L., Ball J.K. (2012). Naturally occurring antibodies that recognize linear epitopes in the amino terminus of the hepatitis C virus E2 protein confer noninterfering, additive neutralization. *J. Virol.* **86** (5), 2739-2749.

Tong C.Y., Gilmore I.T., Hart C.A. (1995). HCV-associated liver cancer. *Lancet.* **345** (8956), 1058-1059.

Wakita T., Pietschmann T., Kato T., Date T., Miyamoto M., Zhao Z., Murthy K., Habermann A., Krausslich H.G., Mizokami M., Bartenschlager R., Liang T.J. (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11** (7), 791-796.

Yu M.Y., Bartosch B., Zhang P., Guo Z. P., Renzi P.M., Shen L.M., Granier C., Feinstone S.M., Cosset F.L., Purcell R.H. (2004). Neutralizing antibodies to hepatitis C virus (HCV) in immune globulins derived from anti-HCV-positive plasma. *Proc. Natl. Acad. Sci USA.* **101** (20), 7705-7710.

Zhang P., Zhong L., Struble E.B., Watanabe H., Kachko A., Mihalik K., Virata-Theimer M.L., Alter H.J., Feinstone S., Major M. (2009). Depletion of interfering antibodies in chronic hepatitis C patients and vaccinated chimpanzees reveals broad cross-genotype neutralizing activity. *Proc. Natl. Acad. Sci. USA.* **106** (18), 7537-7541.

Zhou Y., Bartels D.J., Hanzelka B.L., Muh U., Wei Y., Chu H.M., Tigges A.M., Brennan D.L., Rao B.G., Swenson L., Kwong A.D., Lin C. (2008). Phenotypic characterization of resistant Val36 variants of hepatitis C virus NS3-4A serine protease. *Antimicrob. Agents Chemother.* **52** (1), 110-120.