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Neutralizing antibodies in patients with chronic hepatitis C infection treated with (Peg)-interferon/ribavirin

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Abstract

Background: The role of neutralizing antibody (NAb) in determining response to antiviral therapy has not been established.
Objective: In this study we have analysed the kinetic's of the NAb response in patients with chronic hepatitis C who received antiviral therapy.
Study design: Seventeen patients infected with genotype 1, 2a/c or 3a hepatitis C virus (HCV) were enrolled, eight with a sustained virological response (SVR), five non-responders and four relapsers.
Results: The mean NAb titre required to neutralize 50% of the E1E2-pp in patients who achieved an SVR ($294 \pm$ S.D. 51), in relapsers ($246 \pm$ S.D. 61.7) and non-responders ($286 \pm$ S.D. 80.95) did not differ significantly between the patient groups and did not alter during the course of treatment ($P > 0.01$). Genetic variation present before antiviral therapy was analysed by single strand conformation polymorphism (SSCP) and failed to demonstrate a significant difference in the mean number of amplified E1E2 DNA fragments from the serum of patients who achieved an SVR ($3.15 \pm$ S.D. 1.53), relapsers ($2.8 \pm$ S.D. 1.32) or non-responders ($3.69 \pm$ S.D. 1.75). The baseline serum HCV viral loads were also not significantly different between patients who achieved an SVR (1.4×10^6 copies/ml; $\pm$S.D. 2.4×10^6), relapsers (1.3×10^7 copies/ml; $\pm$S.D. 2.4×10^7) and non-responders (1.5×10^6 copies/ml; $\pm$S.D. 1.1×10^6).
Conclusion: We have shown that neutralizing anti-HCVpp antibody is not associated with response to antiviral therapy. In addition, there was no correlation between baseline virological load, circulating viral quasispecies, NAb titres and final response to treatment.

Keywords: Liver disease; Antivirals; Viral hepatitis; Humoral immunity

Abbreviations: Nab, neutralizing antibody; HCV, hepatitis C virus; SVR, sustained virological response; SSCP, single strand conformation polymorphism; HCVpp, HCV pseudotype particles; ISDR, interferon sensitivity determining region; HCV-LPs, HCV-like particles

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1. Introduction

Hepatitis C virus (HCV) is a human pathogen of major global significance and currently treatment is confined the combination of pegylated interferon-α and ribavirin (Fried et al., 2002; Hadziyannis et al., 2004; Manns et al., 2001).

Clearance of HCV infection is associated with the development of an early broad and persistent class 1 restricted CD8+ CTL (Lechner et al., 2000; Missale et al., 1996) and CD4+ T cell (Diepolder et al., 1997; Lamonaca et al., 1999) responses. Neutralizing antibody (NAb) responses have an important role in the prevention of infection and in limiting viremia (Bartosch et al., 2003; Eren et al., 2006; Hsu et al., 2003; Lavillette et al., 2005; Youn et al., 2005; Yu et al., 2004). In addition, patients chronically infected with HCV have relatively high titres of cross-reactive NAb (Bartosch et al., 2003).

Humoral and both intrahepatic and peripheral cellular immune responses are important in determining response to antiviral therapy with interferon and ribavirin (Baumert et al., 2000; Cramp et al., 2000; Vrolijk et al., 2003). Patients with chronic hepatitis C infection also have high titres of antibodies to envelope proteins of HCV-like particles (HCV-LPs) (Baumert et al., 2000). However, the role of NAb in determining response to antiviral therapy has not been established.

In this study we have analysed the kinetic's of the NAb response in patients with chronic hepatitis C who received treatment with interferon/pegylated interferon and ribavirin. We show that NAb levels do not alter during or after treatment in patients undergoing antiviral therapy and that there were no differences in baseline viral quasispecies diversity between groups. In addition, no differences were observed between groups of patients who had an sustained virological response (SVR), those who relapsed following treatment and patients who did not respond to therapy.

2. Materials and methods

2.1. Cell lines and culture

293T human embryo kidney cells and Huh7 human hepatoma cells were maintained in Dulbecco's minimal essential medium containing 10% fetal calf serum and 2 mM L-glutamine (DMF10).

2.2. Patient serum/plasma samples

Patients attending the Hepatitis Clinic at the Victorian Infectious Diseases Service, Royal Melbourne hospital and undergoing treatment for chronic hepatitis C infection with either interferon or pegylated interferon together with ribavirin were enrolled (Table 1). Serum or plasma samples were collected from patients before commencement of treatment and at regular intervals throughout the course of therapy. HCV viral loads were determined using the Versant HCV RNA 3.0 bDNA assay (Bayer HealthCare, Tarrytown, NY) according to the manufacturer's instructions. All patients were serologically negative for hepatitis B virus and human immunodeficiency virus.

SVR's were defined as those with a negative HCV RNA 6 months after cessation of treatment, non-responders as those who failed to have more than a 2 log 10 reduction on HCV viral load after 12 weeks of treatment and relapsers as those had negative HCV RNA during treatment but who became HCV RNA positive 1 month after cessation of treatment.

2.3. Plasmids and vectors

Construction of the pCDNA4HisMax (Invitrogen, Carlsbad, CA)-based vector, pE1E2H77c, has been described previously (Drummer et al., 2003). The plasmid pHEF-VSVg and the HIV-1 luciferase reporter vector pNL4-3.LUC.R$^-$E$^-$ were obtained from Dr. N. Landau through the NIH AIDS Research and Reference Reagent program.

2.4. Radioimmunoprecipitation and E1E2-pp neutralizing antibody assays

Radioimmunoprecipitation was performed using 293T cells transfected with 2 μg of pE1E2H77c as described previously (Drummer et al., 2003). The production of E1E2/HIV pseudotyped particles has also been described previously (Drummer et al., 2003). For neutralization assays the 50% neutralizing antibody titre (NAb_{50}) was calculated by determining the mean serum dilution required to reduce entry of E1E2-pp by 50% compared to entry of E1E2-pp in the absence of serum. Only antibodies mediating greater than 50% inhibition of E1E2-pp infectivity were considered positive and NAb_{50} titres below the lowest dilution of serum tested are shown as <100. Serum and plasma samples were also tested at the lowest dilution for non-specific inhibition mediated by antibody or interferon using VSV-G pseudotyped HIV-1 particles. None of the serum or plasma tested inhibited the infectivity of VSV-pp (data not shown). Positive (PCR+ Ab+) and negative serum (PCR− Ab−) serum was obtained from the National Serum Reference Laboratory.

2.5. Single strand conformation polymorphism

Single strand conformation polymorphism (SSCP) of HCV E1E2 region was performed as described by Laskus and coworkers (Laskus et al., 2004). HCV RNA was extracted from patient serum samples using a QIAamp Viral RNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. HCV RNA was reverse transcribed to cDNA using the C/E2 external reverse primer and SuperScript II Reverse Transcription kit (Invitrogen) according to the manufacturer's instructions.

Nested PCR was used to amplify a 331-bp DNA product from E1E2 (HCV nt 954–1285). The first round reaction mix contained 10 μl of cDNA tem-

plate, 4 μl of Red Taq 10× Reaction Buffer (Sigma), 4 μl of 10 mM dNTP mix (New England Biolabs), 3.2 μl of 25 mM $MgCl_2$, 50 pmol each of C/E2 external forward (gCgTCCgggTTCTggAAg ACggCgTgAACTATg-CAACAgg) and reverse (AggCTTTCATTgCAgTTCAAg-gCCgT gCTATTgATgTgCC) primers (Geneworks, Adelaide, Australia), 1.5 μl of Red Taq DNA Polymerase (Sigma) and 15.3 μl of sterile water to a final volume of 40 μl. Initial denaturation was at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 20 s and extension at 72 °C for 45 s. A final extension was performed at 72 °C for 5 min.

An aliquot (0.5 μl) of the first round reaction mix was added to the nested PCR reaction mix together with 4 μl of JumpStart REDTaq 10× Reaction Buffer (Sigma–Aldrich, St. Louis, MO), 4 μl of 10 mM dNTP mix (New England Biolabs), 3.2 μl of 25 mM $MgCl_2$, 50 pmol of E1E2 nested forward (ggCATgggATATgATgATgAACTggTC CCCTAC) and reverse (AgTTCAAggCCgTgCTATTgAT-gTgCCAACTggCCgTTggT) primers (Geneworks), 1.5 μl of JumpStart REDTaq DNA Polymerase (Sigma–Aldrich) and 39.2 μl of sterile water. The reaction conditions were the same as in the first round PCR.

The E1E2 PCR products were purified using a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions and resolved by electrophoresis in a 2% agarose (Scientifix, Melbourne, Australia) and ethidium bromide staining. The DNA band of the correct size was excised, purified with a NucleoSpin Extract II kit (Macherey-Nagel, Duren, Germany) and single stranded DNA released by boiling at 97 °C for 3 min followed by quenching on ice. Denatured E1E2 DNA was separated on a 8% denaturing polyacrylamide gel containing 25% formamide (Calbiochem, Merck, Germany), stained with ethidium bromide and the number of individual bands in respective duplicate samples counted.

3. Results

3.1. The ability of patient antibodies to recognize HCV E1E2 glycoproteins

Patients enrolled in this study were infected with either genotype 1, 2a/c or 3a viruses. Of 17 patients enrolled, 8 had SVR, 5 failed to respond to therapy (non-responders) and 4 patients responded to therapy but relapsed soon after completing treatment (relapsers) (Table 1).

NAb responses in patient plasma/serum specific to intracellular non-covalent E1E2 heterodimers (the precursors to the forms incorporated into E1E2-pp's) were initially detected using radioimmunoprecipitation (Drummer et al., 2003; Keck et al., 2004). Antibodies specific to non-covalent E1E2 heterodimers were detected in 6/8 patients who achieved an SVR, 5/5 non-responders and 4/4 post-treatment failure patients at all times during and post-treatment (Fig. 1; Table 2). Antibody to E1E2 was detected at week 13 of treatment but not at week 24 in patient 7 who had an SVR (Table 2). One patient with a SVR infected with a genotype 1 virus (patient 4) did not have detectable antibody to E1E2 at any time (Table 2).

3.2. Neutralizing antibody response of HCV infected patients in response to treatment

In order to examine how the NAb response of patients undergoing treatment for chronic HCV infection altered in response to therapy we examined the ability of patient serum

Table 1
Baseline virological and treatment regimens of patients with sustained virological response (SVR), relapse and non-response to antiviral treatment

Patient groups	HCV genotype	HCV viral load (copies/ml)	Liver histology	Treatment regimen and duration
SVR				
1	3a	7.2×10^6	G2S2	P/R, 24 wk
2	3a	4.4×10^5	G4S4	I/R, 24 wk
3	3a	3.7×10^5	G3S3	I/R, 48 wk
4	1a	3.5×10^4	G2S2	I/R, 48 wk
5	1a	1.2×10^6	G2S2	P/R, 48 wk
6	2a/c	1.5×10^5	G2S2	I/R, 24 wk
7	3a	1.1×10^6	G2S4	P/R, 48 wk
8	1	8×10^5	G2S4	I/R, 48 wk
Relapsers				
1	1a/b	7.6×10^6	G2S2	P/R, 48 wk
2	1a	8.4×10^5	G1S2	P/R, 48 wk
3	3a	1.2×10^6	G3S4	P/R, 48 wk
4	1a	48.9×10^6	G2S2	P/R, 48 wk
Non-responders				
1	1	1.1×10^6	G3S2	P/R, 48 wk
2	1a	20.1×10^6	G1S2	I/R, 48 wk
3	3a	5.8×10^5	G3S4	P/R, 48 wk
4	1	32×10^6	G2S4	P/R, 48 wk
5	1	7.8×10^5	G3S3	I/R, 48 wk

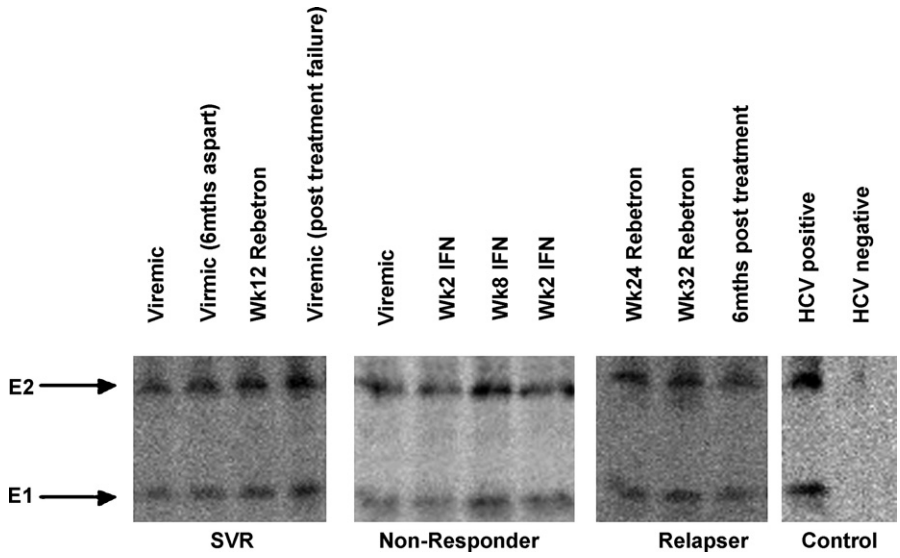

Fig. 1. Ability of patient antibodies to immunoprecipitate E1E2 heterodimers. Serum or plasma collected from patients was used to immunoprecipitate E1E2 heterodimers from ^{35}S-Met/Cys labeled E1E2 transfected cell lysates prior to SDS-PAGE on 10–15% gradient gels and phosphorimage analysis. A representative patient of each group is shown and corresponds to, sustained virological response (patient 5, SVR group), non-responder (patient 17, NR group) and relapse following treatment (patient 1, REL group). A reference HCV positive plasma and HCV negative plasma is shown and the location of E2 and E1 indicated by arrows. HCV, hepatitis C virus; NR, non-responder; REL, relapser.

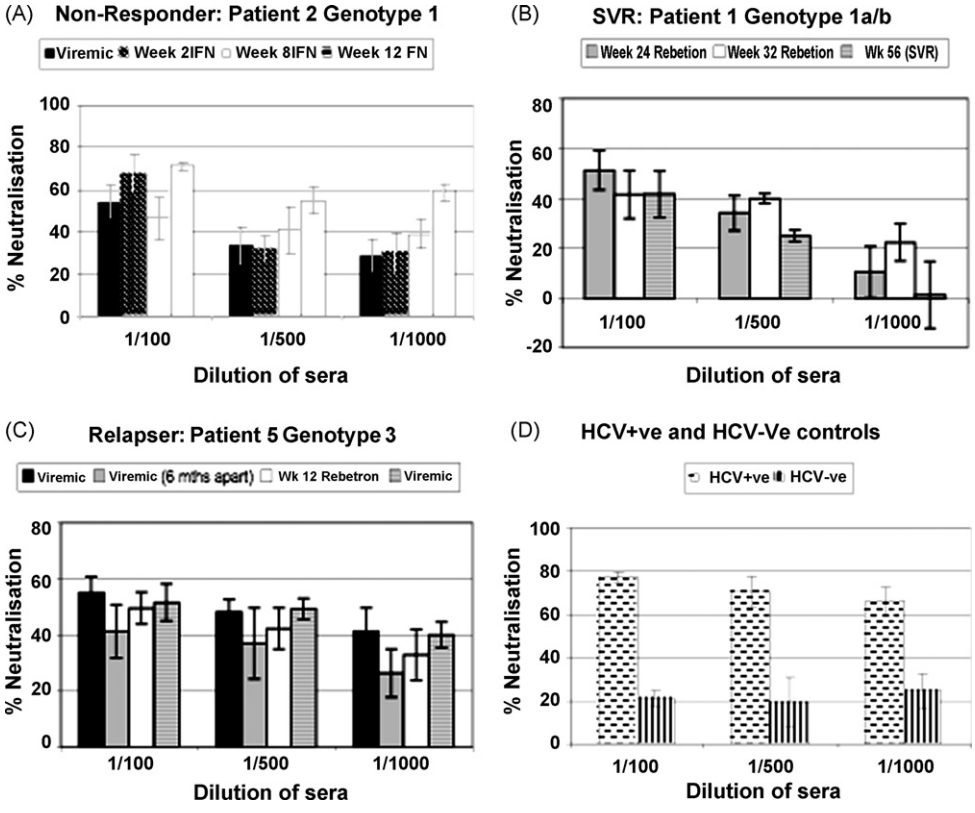

Fig. 2. Ability of patient plasma/sera to neutralize infectivity of genotype 1a E1E2-pseudotyped particles. Representative antibody mediated neutralization of serum/plasma from a patient with a SVR to HCV treatment (A), a non-responder to HCV treatment (B), a patient with a relapse of HCV following cessation of treatment (C) and control plasmas from a patient with chronic HCV infection and an HCV negative patient (D) at dilutions of 1:100, 1:500 and 1:1000. Serum/plasma was incubated with E1E2-pp for 1 h before addition to Huh7 cells. Following 3 days incubation luciferase activity in cell lysates was quantitated in a luminometer. Data is expressed as the mean percentage neutralization and standard deviation relative to infectivity determined in the absence of serum/plasma. SVR, sustained virological response; HCV, hepatitis C virus.

Table 2
Summary of IP and NAb results

SVR patient code	HCV Gt	Week of treatment or follow up (F/U)	IP[a]	NAb_{50} titre[b]	Non-responder patient code	HCV Gt	Week of treatment or follow up (F/U)	IP[a]	NAb_{50} titre[b]	Relapser patient code	HCV Gt	Week of treatment or follow up (F/U)	IP[a]	NAb_{50} titre[b]
1	3a	Viremic	+	<100	1	1	Viremic	+	488	1	1	24	+	293
		16	+	280			20	+	267			24 (F/U)	+	740
2	3a	Viremic	+	407	2	1a	Viremic	+	<100	2	1a	Viremic	+	<100
		Viremic	+	<100			2	+	566			12	+	<100
		12	+	75			12	+	325			24	+	<100
		24 F/U	+	297			48 (F/U)	+	571			24 (F/U)	+	<100
3	3a	Viremic	+	<100	3	3a	Viremic	+	<100	3	3a	Viremic	+	<100
		12	+	149			14	+	144			36	+	455
		24	−	<100			30	+	356			24 (F/U)	+	547
4	1a	Viremic	−	<100	4	1	Viremic	+	<100	4	1a	Viremic	+	536
		10	−	<100			2	+	1449			4	+	130
		24	−	<100			8	+	329			8	+	<100
		36 F/U	−	<100			12	+	153			19	+	<100
5	1a	Viremic	+	577	5	1	Viremic	+	160			36	+	237
		1	+	618			4	+	<100			48	+	<100
		10	+	498			8	+	<100			2 (F/U)	+	604
		12	+	566			16	+	<100					
		14	+	<100			26	+	<100					
		44	+	150			32							
6	2a/c	Viremic	+	<100										
		4	+	<100										
		16	+	<100										
		20	+	184										
		48	+	<100										
7	3a	Viremic		400										
		10		642										
		14		78										
		24		546										
8	1	Viremic		696										
		4		615										
		12		773										
		20		1210										
		26		307										

[a] Ability of serum/plasma to immunoprecipitate non-reduced forms of E1E2 from transfected and radiolabelled cell culture lysates. +: E1 and E2 detected after phosphorimage analysis. −: no E1 or E2 detected after phosphorimage analysis.

[b] Mean dilution of serum required to obtain 50% neutralization of E1E2-pp.

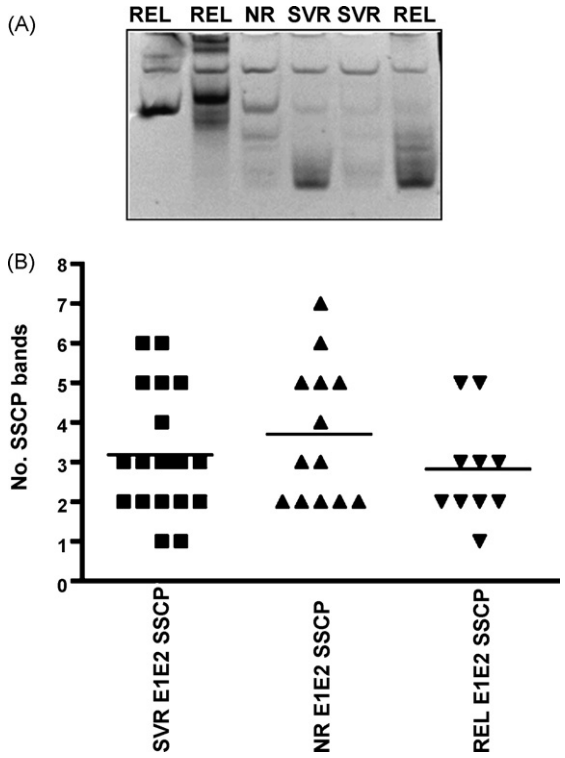

Fig. 3. SSCP analysis of viral variants in E1E2. (A) Representative gel of amplified E1E2 PCR products from viremic patient sera separated on a denaturing polyacrylamide gel viral quasispecies stained with ethidium bromide. Each lane represents the viral quasispecies present in a single infected individual and each band represents a viral variant. (B) Scatter plot of the number of SSCP bands in individual patient sera in duplicate together with the mean number of bands. SSCP, single strand conformation polymorphism; PCR, polymerase chain reaction; SVR, sustained virological response; NR, non-responder; REL, relapser.

or plasma collected at various times during and after treatment to neutralize E1E2-pp's. We only examined the binding of sera to genotype 1a E1E2-pp alone as previous studies have demonstrated that in chronic HCV infection broadly cross-reactive antibodies are elicited that are able to neutralize diverse HCV genotypes including the prototype HCV strain H77c (Meunier et al., 2005). In addition, neutralization of E1E2-pp's was performed using serum rather than IgG as we have only observed enhancement of E1E2-pp infectivity with less than 5% of normal human serum tested (results not shown). In general, with the exception of relapser patient 2, the detection of NAb at one time point correlated with the presence of antibody that immunoprecipitated E1E2 glycoproteins (Fig. 2). The mean dilution of antibody required to neutralize 50% (NAb_{50} titre) of the E1E2-pp in patients who achieved an SVR ($294 \pm$ S.D. 51), in relapsers ($286 \pm$ S.D. 80.95) or those patients who did not respond to antiviral treatment ($246 \pm$ S.D. 61.7) did not alter during the course of treatment compared to the 50% NAb titre in serum/plasma collected at the end of treatment or up to 9 months following treatment cessation ($P > 0.01$) (Table 2). The mean 50% NAb titres in each patient group were also not significantly different, reflecting the lack of correlation between the levels of NAb and response to treatment with common HCV therapies.

3.3. Single strand conformation polymorphism (SSCP) of E1E2

The degree of baseline variation in a 331 base pair region of E1E2 (HCV nt 954–1285) was determined using SSCP. Multiple bands were evident in individual patient samples consistent with viral genomic sequence variation (Fig. 3A). The mean number bands of amplified E1E2 DNA fragments from the serum of patients who achieved an SVR (mean $3.15 \pm$ S.D. 1.53) did not differ significantly from patients who relapsed (mean $2.8 \pm$ S.D. 1.32) or who did not respond (mean $3.69 \pm$ S.D. 1.75) to antiviral treatment (Fig. 3B). The baseline serum HCV viral loads were also not significantly different between patients who achieved an SVR (1.4×10^6 copies/ml; $\pm$S.D. 2.4×10^6) compared to relapsers (1.3×10^7 copies/ml; $\pm$S.D. 2.4×10^7) and non-responders (1.5×10^6 copies/ml; $\pm$S.D. 1.1×10^6).

4. Discussion

In this study we have shown that neutralizing anti-HCVpp antibody is not associated with sustained response or a failed response to antiviral therapy with interferon/pegylated interferon and ribavirin. We also found that the NAb titres were similar in patients infected with different HCV genotypes and did not change with time through the course of antiviral therapy. In addition, there was no correlation between baseline virological load or circulating viral quasispecies, NAb titres and final response to treatment.

The role of humoral responses in clearance of HCV is uncertain. Strong peripheral and intrahepatic $CD8^+$ T-cell responses are important in determining an SVR in response to treatment with interferon and ribavirin (Lasarte et al., 1998; Lauer et al., 2005). In contrast to CD8+ T-cell responses, the role of humoral responses in determining effectiveness to interferon and ribavirin remains unclear.

Baumert and coworkers have reported the presence of high titres of antibodies to HCV-LPs in the serum of patients who achieved an SVR to interferon/ribavirin therapy in contrast to treatment relapsers and non-responders (Baumert et al., 2000). It was suggested that anti-HCV-LP antibodies may serve a useful role in predicting treatment outcome however they did not investigate the role of NAb in interferon responsiveness (Baumert et al., 2000).

The presence of high pre-treatment viral genomic diversity (Ueda et al., 2004) and the early expansion of the viral quasispecies population after the onset of therapy have both been linked to reduced effectiveness of antiviral therapy (Farci et al., 2002). In order to investigate a possible role of NAb in limiting pre-treatment viral quasispecies and thereby contributing to the effectiveness of interferon we examined viral diversity in baseline sera. We found that in baseline viremic

sera there is no significant difference between the number of viral variants in the E1E2 region and individual responses to therapy.

Although it has been proposed that anti-E2 antibody may serve a role in controlling HCV viremia (Youn et al., 2005) we did not find a significant difference in the NAb titre or baseline viral load and viral diversity in patients who achieved an SVR in comparison to those who had failed antiviral therapy. Alternatively, the neutralization assay used here which only detects cross-reactive antibodies capable of neutralizing one strain of HCV pseudotyped particles may not be sufficiently sensitive to detect subtle, isolated specific changes to NAb over the course of treatment. A follow up longitudinal study now aims to determine the strain specific NAb response in patients undergoing pegylated interferon treatment.

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