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**A PEPTIDE MIMOTOPE OF HEPATITIC C VIRUS E2 PROTEIN
IS IMMUNOGENIC IN MICE AND BLOCK HUMAN ANTI-HCV
SERA**

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Keywords:	KEY WORDS: HCV; mimotope; therapeutic vaccine.



Table I. Phage yields obtained from phage display peptide library after three rounds of biopanning using/with mAbs 3e5-1 and 5E5/H7.

Round	Incubation time	3e5-1			5E5/H7		
		Input (TU/ml)	Output (TU/ml)	% yield	Input (TU/ml)	Output (TU/ml)	%yield
1	overnight	1×10^9	2.2×10^4	0.001	5.8×10^{10}	3.45×10^4	0.00006
2	4 hrs	8×10^9	9.3×10^6	0.116	2×10^{10}	1.86×10^5	0.0009
3	10 min	1.2×10^9	2.8×10^6	0.230	2×10^{10}	5.8×10^6	0.029

Table II. Assessment of binding anti-mimotope peptide antibodies to E2 protein.

Exp. No.	E2 source	Mock antigen source	Anti-mimotope antibody titre (log ₁₀)	Mean titre (SD)	NMS antibody titre (log ₁₀)	Mean titre (SD)	P value
1	supernatant	PBS	1.72 1.87 2.03	1.87 (0.16)	1.69 1.39 1.3	1.46 (0.20)	0.0492
2	supernatant	PBS	1.675 1.915 2.075	1.88 (0.20)	1.57 1.3 1.39	1.42 (0.14)	0.0292
3	supernatant	PBS	1.97 2.0 2.45	2.14 (0.27)	1.39 1.40 1.41	1.4 (0.01)	0.0089
4	supernatant	PBS	1.675 1.9 2.15	1.91(0.24)	1.6 1.495 1.495	1.53 (0.06)	0.0557
5	E2-cell lysate*	Uninfected cell-lysate	2.0 1.96 2.12	2.02 (0.08)	1.39 1.44 1.43	1.42 (0.03)	0.0003

* COS-7 cell-lysate infected with RAAd-79 expressing E2 protein

Table III. Measurement of inhibition of E2-CD81 interaction in convalescent human HCV sera.

Patient No. (all anti-HCV +ve)	PCR	CD81 in presence of serum (OD _{450nm}) ¹		% inhibition ¹	inhibition status ¹	Reactivity with mimotope K1 peptide
1	+/-	0.02*	0.50**	97%	++	+
2	-ve	0.88	0.87	-	-	++
3	+/-	0.02	0.19	97%	++	-
4	+ve	0.03	0.56	96%	++	+
5	-ve	0.78	0.82	-	-	-
6	+/-	0.11	0.75	86%	++	++
7	+/-	1.11	0.92	-	-	-
8	-ve	0.83	0.82	-	-	-
9	+/-	0.02	0.22	97%	++	+
CBH-7 [£]	NA	0.11		86%	++	NA
CBH-9 [£]	NA	1.07		-	-	NA

* Sera were tested at a final dilution of 1/200

** Sera were tested at a final dilution of 1/1000

[£] CBH-7 and CBH-9 are two human mAbs (final 2.5µg/ml), used as positive and negative control respectively

NA: not applicable

¹ net antibody value is given after subtraction of value obtained for E2 GST-agm EC2.

+/-: weak positive PCR

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For Peer Review

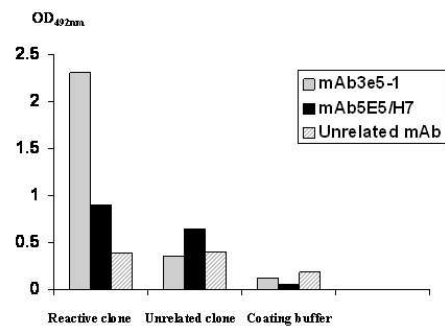


Figure 1. Analysis by ELISA of phage clone selected by biopanning. The mAb 3e5-1 showed stronger binding to its reactive phage clone with high degree of discrimination to unrelated clones compared to mAb 5E5/H7. No binding was observed using an unrelated antibody to either of the clones.
254x190mm (96 x 96 DPI)

Mimotope	F	G	E	F	S	F	Q	Y	D	C	S	S	W	G	C									
Heptapeptide						53	G	A	P	T	Y	S	W	G	52									
(1a) HCV-H		T	T	D	R	S	G	A	P	T	Y	S	W	G	A	N	D	T	D	V	F	V	51	
(1a) HCV-1															E									
(1a) HC-J1												<i>N</i>	.	.	.									
(1b) HCV-T						F						.	.	.	E		E					L	L	
(1b) HCJ						F						.	.	.	E		E					L	L	
(1b) HC-J4								V				.	.	.	E		E					M	L	
(1b) HC-JT												<i>N</i>	.	.	.		E					L	L	
(2a) HC-JK1						F		V				<i>N</i>	.	.	.	D		E				L	L	
(2b) HCV-BK						F		V				<i>R</i>	.	.	.	E		E				L	L	
(3a) NZL1						A	R	V				<i>F</i>	.	.	.	E		E	K			L	L	
(4a) L835						H	L	V				<i>F</i>	.	.	.	E		E	S			L	L	
(5a) UH1480						R		C				<i>N</i>	.	.	.	S		E				I	L	L
(6) GZ52557						Q	R	L				<i>F</i>	.	.	.			K				L	L	

Figure 2. Delineation of the epitope mimicked by mimotope K1 peptide. Alignment of the mimotope K1, peptide 523GAPTYSWG529 and amino acid sequence corresponding to 513-531 residues of the native E2 of HCV prototype H, strains in subtypes 1a, 1b, 2a, 2b, 3a, 4a, 5a and 6. HCV strains protein accession numbers are as follows HCV-H.{AAA45534}; HCV-1 {AAA45676}; HC-J1 {BAA01582}; HCV-T {ABU97068}; HCV-J {BAA14233}; HC-J4 {BAA00706}; HC-JT {ABU97068}; HC-JK1 {CAA43793}; HCV-BK {AAA72945}; HC-J6 {BAA00792}; HC-JB {BAA01761}; NZL1{YP_001469631}; L835 {ABD758321}; EUH1480 {YP_001469633}; GZ52557 {AAV75597}. Residues shown in bold are identical to the native 513-531 sequence. Residues in italic indicate conservative changes. The common motif shared by the mimotope K1, peptide 523GAPTYSWG529 and the HCV E2 sequence is boxed. Dots indicate identical amino acids to the prototype HCV-H strain.

254x190mm (96 x 96 DPI)

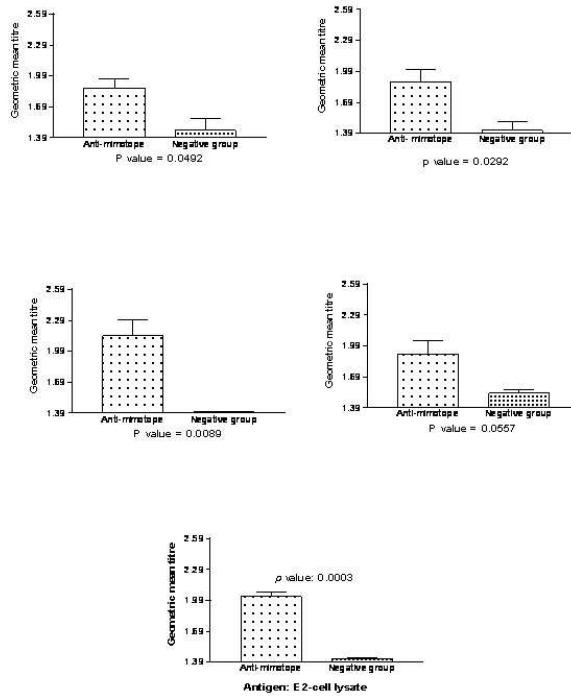


Figure 3. Assessment of binding the anti-mimotope antibodies to the native E2 protein. Anti-mimotope antibodies were tested by ELISA for reactivity to either partially purified E2 protein or E2 cell lysates. Results were expressed as geometric mean titre + SD.
254x190mm (96 x 96 DPI)

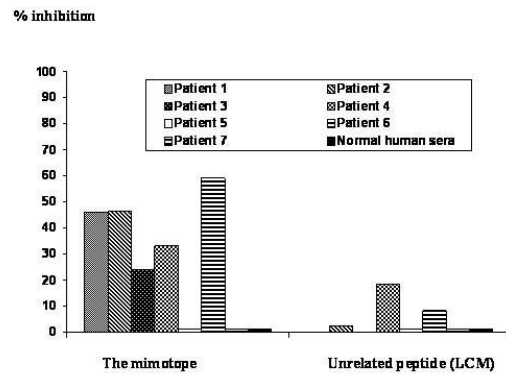


Figure 4. Competitive inhibition ELISA using mimotope K1 peptide and selected human sera positive for anti-E2 antibodies. Inhibition was measured as a percentage of the direct binding of the human anti-E2 antibodies to the E2 protein.

254x190mm (96 x 96 DPI)

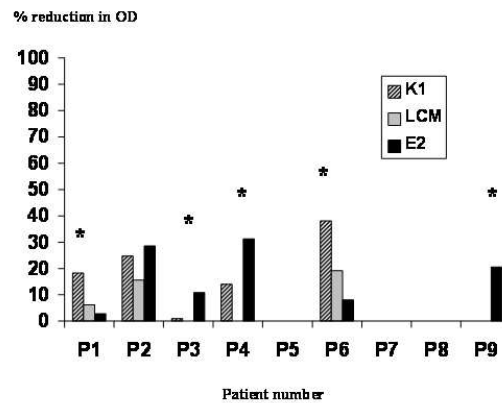


Figure 5. Competitive ELISA using the mimotope K1 peptide and the recombinant E2 protein. Competition was measured as the inhibition exerted by mimotope K1 peptide competing with E2 protein for blocking the binding of human sera E2 protein-coated plates. Competition was calculated as a percentage of the direct binding to the E2 protein-coated plates. The mimotope competed with the recombinant E2 protein in four sera. Asterisks indicate sera with the capacity to inhibit E2-CD81 interaction.

254x190mm (96 x 96 DPI)

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ABSTRACT

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10 3
11
12 4 Conformational B-cell epitopes on the HCV E2 protein recognised by human antibodies
13
14 5 were characterised by use of a peptide mimotope named K1. K1 was identified by two
15
16 6 HCV anti-E2 monoclonal antibodies (mAbs) following selection and purification of phage
17
18 7 clones containing a 15-mer random peptide insert. Murine antisera to the mimotope K1
19
20 8 recognised the E2 protein. Five of eight human sera from patients who had cleared HCV
21
22 9 recognised the K1 mimotope. Binding to E2 in four individuals with the capacity to block
23
24 10 E2- CD81 interaction was inhibited by the mimotope K1. The results demonstrate that
25
26 11 anti-E2 antibodies in sera from patients who have cleared HCV infection are directed
27
28 12 against a conformational B-cell epitope on E2 that can be mimicked with linear synthetic
29
30 13 peptides. These findings could have implications for vaccine design by employing linear
31
32 14 mimotopes to direct B-cell responses against those specific E2 epitopes that may correlate
33
34 15 with immunity. (Word count: 144)

1 **KEY WORDS:** HCV; mimotope; therapeutic vaccine.
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10 **INTRODUCTION**

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14 Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. Twenty percent
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16 of chronically infected individuals develop liver cirrhosis that is linked to hepatocellular
17
18 carcinoma in many regions [Kuo et al., 1989; Saito et al., 1990]. However, there is no
19
20 protective vaccine against HCV infection [Houghton and Abrignani, 2005]. Gamma
21
22 interferon and ribavirin, are currently the only antiviral agents for therapy that are
23
24 successful in 50% of treated patients [Ahn and Flamm, 2004]. Therefore, the development
25
26 of new antiviral agents or therapeutic vaccines is essential.
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30 HCV contains a 9.6kb positive-stranded RNA genome with considerable homology to
31
32 other members of the family *flaviviridae* [Miller and Purcell, 1990]. The genome encodes
33
34 two envelope proteins E1 and E2 which are important candidate antigens for a vaccine
35
36 against HCV. Both glycoproteins show substantial amino acid sequence variation with a
37
38 hypervariable region (HVR1) located at the N-terminal region of E2 [Hijikata et al., 1991;
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40 Kato et al., 1992; Weiner et al., 1991].
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48 In chimpanzees, immunisation with recombinant E1/E2 partially protects against
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50 homologous virus challenge [Choo et al., 1994] and antibodies to the E2 protein correlated
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52 with reduction in virus load but not from reinfection [Farci et al., 1992; Lanford et al.,
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54 2004; Youn et al., 2005]. In persons infected with HCV, the presence of neutralising
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56 antibodies against HCV E2 correlates with virus clearance [Lavillette et al., 2005; Pestka
57
58 et al., 2007].
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5 1 Several cellular molecules have been identified as putative receptors for HCV. The CD81
6
7 2 tetraspanin, scavenger receptor class B type I (SR-BI) and the recently reported co-
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9 3 receptor; Claudin-1 are the most promising candidates [Evans et al., 2007; Pileri et al.,
10
11 4 1998; Scarselli et al., 2002]. Studies using assays of retroviral pseudoparticles (HCVpp)
12
13 5 and infectious clones support a central role for CD81 in mediating viral infection
14
15 6 [Bartosch et al., 2003b; Cormier et al., 2004; Lavillette et al., 2005; Lindenbach et al.,
16
17 7 2005]. Furthermore HCV E2 has been shown to interact with the large extra-cellular loop
18
19 8 (LEL) of CD81 on human cells [Evans et al., 2007; Pileri et al., 1998; Scarselli et al.,
20
21 9 2002]. The capacity of antibodies to inhibit E2 binding to CD81 had been used previously
22
23 10 for detecting the neutralising capacity of antibodies against (Nt Ab) HCV [Flint et al.,
24
25 11 1999]. These neutralising antibody titres were reported to correlate with spontaneous
26
27 12 resolution of chronic infection [Abrignani, 1997; Bartosch et al., 2003a]. Such Nt Ab is
28
29 13 likely to be directed against conformational B-cell epitopes of E2, which appear to be
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31 14 conserved between different genotypes [Habersetzer et al., 1998; Hadlock et al., 2000;
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33 15 Owsianka et al., 2006].
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43 17 The E2 protein model based on the envelope protein of tick borne encephalitis virus
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45 18 proposed two CD81 binding regions 1 and 2 on E2 protein [Yagnik et al., 2000].
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47 19 Therefore, blocking the binding of HCV to CD81 would be of great value in the quest for
48
49 20 a vaccine development. In this study two anti-E2 mAbs, 3e5-1 and 5E5/H7, were
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51 21 employed to select mimotopes that are involved in protection, neutralising activity using a
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53 22 15-mer random phage displayed peptide library. mAb 3e5-1 and 5E5/H7 were of
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55 23 considerable interest. The conformational mAb 5E5/H7 was produced initially to
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57 24 intracellularly envelope protein hence epitopes(s) recognised by this antibody would
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59 25 display enhanced receptor-binding ability as would the E2 native form. The anti-linear
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1 mAb3e5-1, however, was produced to E2 truncated form and recognised secreted and
2 intracellularly forms of E2, suggesting possible shared epitope recognition as mAb
3 5E5/H7 (Houghton M, personnel communication; invention EP0980434B1
4 (<http://www.freepatentsonline.com/EP098043B14.html>)). Additionally, mAb 5E5/H7 was
5 initially used to affinity purify the E1/E2 proteins used in the protection study in
6 chimpanzees where considerable protection was achieved against homologous challenge
7 [Choo et al., 1994; Rosa et al., 1996]. As such, epitope recognised by this antibody could
8 block viral infectivity. The mimotope was tested for its ability to bind to human antisera to
9 HCV, to block E2- CD81 interaction and to raise antibodies to HCV in mice. The
10 identified mimotope could have potential applications for the design of an
11 immunotherapeutic vaccine that would target the CD81-HCV E2 interaction site(s).

MATERIALS AND METHODS

Monoclonal Antibodies

16 Two HCV E2 specific mAbs, 3e5-1 and 5E5/H7, were used in this study. Both were
17 kindly provided by Dr. M. Houghton, Chiron Corporation, USA. mAb 3e5-1(immunogen
18 Insect E2, aa 404-661) is directed against a linear epitope on the secreted and intracellular
19 E2, whereas, the conformational mAb 5E5/H7 (Hella E1/E2, aa 1-967) is thought to
20 recognises intracellularly expressed conformational epitope [Houghton M Invention
21 EP0980434B1(<http://www.freepatentsonline.com/EP0980434.html>)]. The anti-E2
22 conformational mAb 12D11 was generously given by Dr. Geert Maertens (Innogenetics,
23 Gent, Belgium (<http://www.freepatentsonline.com/EP1845108A2.html>)) and used as a
24 positive control in some ELISAs. A mAb Tac specific for Tacaribe virus was used as an
25 unrelated negative control. Two human mAbs the CBH 7 and CBH 9 with characterised

1 E2-CD81 interaction capacity were sourced by Prof. Jane McKeating and used as a
2 positive and negative control, respectively in E2-CD81 binding assays. CBH-7 has
3 neutralisation activity to multiple HCV genotypes recognises conformational epitope on
4 the HCV E2 domain C proposed by Keck et al. [Hadlock et al., 2000; Keck et al., 2005;
5 Keck et al., 2004; Owsianka et al., 2008]. CBH-9 was negative in Nt Ab assay (patent
6 application titled: prevention and treatment of HCV infection employing antibodies
7 directed against conformational and linear epitopes
8 (<http://www.faqs.org/patents/app/20090202482>).

Human Sera

11 A panel of 25 sera was generously supplied by J. Craske, at the Public Health
12 Laboratories, Manchester, UK. The samples were obtained from a longitudinal study of
13 patients infected previously with HCV through the use of contaminated blood clotting
14 factors. All sera were negative for HBV and HIV. Evidence of HCV infection was shown
15 by the presence of anti-HCV antibodies (Ortho Diagnostics) and confirmed by RIBA-II.
16 Only sera containing anti-E2 antibodies were used in this study. Sera were divided into
17 those positive or weak positive for HCV RNA by PCR, (15 patients) and those that did not
18 have demonstrable RNA in their circulation but were positive for anti-HCV antibodies by
19 commercial serological assays (10 patients). Sera from individuals with no history of HCV
20 infection were used as HCV-negative sera (8 individuals).

Screening of a 15-mer Phage Library

23 A random 15-mer peptide library displayed on filamentous phage and *E.coli K91kan* was
24 kindly provided by Dr. G. Smith, University of Missouri, USA. Polystyrene beads (1/4"
25 diameter) (Pierce, Rockford, IL) were coated with 1/1000 dilution of mAb3e5-1 or

1 5E5/H7 in 0.1M carbonate buffer pH9.5 at 4°C, overnight. All other steps were as
2 described before [D'Mello et al., 1997] except that all phage clones were harvested by two
3 successive preprecipitations in 16.7% PEG-8000/3.3M NaCL. The input and output phage
4 were titrated using Luria Broth (LB) media after each round of biopanning, so that the
5 percent enrichment of binding the phage could be calculated. Percent enrichment was
6 calculated as output/input x 100.

8 **DNA Purification And Sequencing Of Phage Clones**

9 The DNA was extracted from the purified phage using the miniprep DNA purification kit
10 (Promega) following the manufacture's procedure. Sequencing of the insert DNA was
11 performed using the AutoRead sequencing Kit (Pharmacia, UK) and following the
12 conditions recommended by the manufacturers. The fluorescence labelled sequencing
13 primer was 5'-FCCCTCATAGTTAGCGTAACG-3' downstream of the oligo insert site of
14 phage DNA. The sequencing reactions were analysed on the ALF automated DNA
15 sequencer (Pharmacia, UK) using a 6% Sequa gel XR (National Diagnostics, UK).

17 **Synthetic Peptides**

18 The peptide mimotope K1 and the chimeric peptide (K1-MVF) containing a T-helper cell
19 epitope from the fusion protein of measles virus [Partidos and Steward, 1990; Partidos and
20 Steward, 1992] were synthesised using the Millipore 9050 synthesiser and Fmoc
21 chemistry as described by D'Mello et al [1997]. Peptides were characterised by high-
22 performance liquid chromatography (HPLC), mass spectrometry and amino acid
23 sequencing. Mimotope K1 Peptide and the chimeric peptide had a single major peak with
24 a retention time of 5.7 and 20.79 minutes and molecular mass of the expected values of
25 1763 and 3309, respectively. The composition of the peptides was also verified by amino

1 acid analysis. A synthetic peptide representing an epitope from the lymphocytic
2 choriomeningitis virus (LCM) was used as an unrelated negative control peptide.

4 E2 Protein

5 Recombinant adenovirus RAdCMV-E2 (RAD-79) expressing E2 protein of HCV-BK type
6 1b (aa 368-693) [Takamizawa et al., 1991] and Adenovirus expressing β -galactosidase
7 (RAd-35) were generously provided by Dr. Gavin Wilkinson (University of Wales,
8 College of Medicine, Cardiff). Detailed RAd-79 propagation, E2 purification method and
9 results are described elsewhere (El-Attar et al., in preparation). Briefly, RAd-79 was
10 propagated and titrated in 293 cells. 200pfu/cell of RAd-79 was used to infect COS-7
11 cells. RAd-35 was treated in same way as RAd-79 and used as negative control antigen to
12 allow discrimination between anti-E2 antibody and anti-adenovirus antibody which might
13 exist in human sera. 293 and COS-7 cells were grown in Glasgow's essential medium and
14 Dulbecco's medium respectively. Growth medium was supplemented with 10% foetal calf
15 serum, 100 I.U./ml penicillin, 100 μ g/ml streptomycin and 1% non-essential amino acids
16 (Gibco, BRL). The expression of E2 protein in COS-7 cells, was detected intracellular by
17 immunofluorescent staining using the mAb 5E5/H7 and extracellular by indirect ELISA
18 using the mAb 12D11. 10uM/ml forskolin was added to the maintenance medium to
19 enhance the expression of the E2 protein and the cell monolayers were incubated for three
20 days. The maintenance medium was replaced by serum-free medium and the secreted E2
21 protein was collected after six days. Supernatants from uninfected COS-7 cells were used
22 as a mock antigen to eliminate any irrelevant antibody binding.

23 Partial purification of E2 protein was achieved by first concentrating the cell supernatants
24 approximately 15-fold by ultra filtration using a 10KDa cut-off membrane (YM10,
25 Amicon) followed by successive gel-purification on S- Sepharose in fractionation range of

1 5x10³- 2.5x10⁵ (Pharmacia Biotech) according to the manufacture's protocol. Collected
2 fractions (at a flow rate 4ml/min) were monitored at 280nm and assayed for the presence
3 of E2 protein by employing the mAb 12D11 at 1/800 dilution. The identity of the
4 produced E2 protein was confirmed by western blot using mAb 12D11 and by
5 deglycosylation using the peptide-N-Glycosidase F (Oxford GlcoScience, UK) and
6 following the manufacture protocol. Fractions containing E2 protein were pooled and
7 dialysed versus Phosphate-buffered saline pH 7.4, and stored at -20°C. In some
8 experiments, lysate of COS-7 cells infected with RAd-79 was used as antigen in ELISA.
9 This will be referred to in the text as E2 cell lysate. Cell lysate from uninfected COS-7
10 cells were used as a mock cell lysate to eliminate any irrelevant antibody binding.

12 **Immunisation Of Mice**

13 Female BALB/c mice, 6 to 8 weeks old, were injected subcutaneously with either
14 50µg/mouse mimotope K1 peptide or the chimeric peptide (K1-MVF) in Freund's
15 complete adjuvant (FCA) or without adjuvant (in PBS). Mice were boosted by the same
16 route and with the same amount of peptides in an emulsion (1:1 ratio) with incomplete
17 Freund's adjuvant after 4 weeks. A control group received PBS in Freund's adjuvant.
18 Mice were bled at day 0 and two weekly intervals thereafter.

20 **Antibody Binding Assays**

21 All assays were performed in 96-well plates (Maxisorb, Nunc or Immunolon2, Dynatech)
22 with reaction volumes of 50µl/well except for the blocking and stopping reaction steps
23 where 100µl/well and 25µl was added, respectively. Plates were coated overnight at 4°C
24 (for peptides and phage clones) and at 37°C (for E2 protein). All primary antibody
25 incubations were at 37°C. Secondary antibody was horseradish peroxidase-conjugated

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4 1 goat anti-mouse IgG (Jackson) or rabbit anti-mouse (Dako) diluted at 1/1000 and applied
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6 2 for 1 hour at 37°C. Washing buffer was PBS, pH 7.4 +0.05% Tween-20. Blocking buffer
7
8 3 was PBS, pH7.4 +10% pig sera. ELISA diluent was blocking buffer supplemented with
9
10 4 0.05% Tween-20. The reaction development was detected by the substrate ortho-
11
12 5 phenylenediamine (OPD) from Sigma for 20 minutes at room temperature in the dark and
13
14 6 stopped by 2M H₂SO₄. The developed colour was detected by measuring the absorbance at
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16 7 492nm using Multiscan plus, version 3.0.
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9 **Phage Clones Binding Assay**

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26 10 Plates were coated with an immunoreactive phage clone and unrelated clone at a
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28 11 concentration of 0.75×10^9 phage particles/ml in 50mM carbonate buffer. Unbound protein
29
30 12 was washed away by three washes. Following blocking, plates were washed as described
31
32 13 above. mAbs 3e5-1, 5E5/H7 and unrelated mAb were diluted 1/200 and added in duplicate
33
34 14 to immunoreactive and unrelated phage clones- coated wells and incubated for 3 hours.
35
36 15 Horseradish peroxidase-conjugated goat anti-mouse IgG was added to all wells. Following
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38 16 washing as above, the substrate OPD was added to all wells and the colour was detected as
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40 17 described above.
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19 **Peptide And E2 Protein Binding Assay**

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50 20 Plates were coated with 10µg/ml of mimotope K1, chimeric peptide or unrelated peptide
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52 21 (LCM). For detection of binding to E2 protein, plates were coated with either 20µg/ml of
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54 22 partially purified E2 protein, 0.5mg/ml of E2 cell lysate, mock cell lysate or coating buffer
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56 23 only. The assays were carried out as above except that mouse sera from each group were
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58 24 tested individually and added in duplicate to coated wells at 2 fold serial dilution starting
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60 25 from 1/50. Positive control mAb 12D11 (1/800), 5E5/H7 (1/400) and 3e5-1 (1/400) were

1 included in E2 protein binding assays to insure the validity of the assay. Antibody titres
2 were determined as \log_{10} values of antibody titres obtained 4 weeks after boosting. The
3 cut off value for peptide assays was the mean OD of the highest concentration of the
4 negative group sera + 3SD.

5 For E2 protein assay, OD values obtained with mock cell lysate or coating buffer were
6 subtracted from values obtained with E2 cell lysate or partially purified E2 protein,
7 respectively. The cut off value was calculated as the mean of the corrected OD value of
8 the lowest dilution of the pre-bleed sera + SD.

Human Sera Assays

11 All conditions of ELISA using human sera were similar to those described for mouse sera,
12 with the following exceptions: assays were performed in a category three laboratory. All
13 sera were heat inactivated at 56°C for 30 minutes before use. Primary antibody incubation
14 was at 4°C overnight. Secondary antibody was Horseradish peroxidase-conjugated rabbit
15 anti-human IgG (Dako) diluted 1/8000. Blocking and diluent buffers were PBS, pH 7.4 +
16 0.5% Casein (sigma) + 0.2% Triton X-100. Washing buffer contained PBS, pH7.4 + and
17 1% Tween-20.

18 **a) Anti-E2 antibody responses.** Plates were coated with 0.5mg/ml of E2-lysate, β -
19 galactosidase cell lysate or mock cell-lysate. Human sera were diluted two-fold starting
20 from 1/20 in ELISA diluent and added in duplicates to all three antigen coated plates. In
21 this assay 0.5% of mock antigen was incubated in the diluent to minimise the background.
22 The plates were then incubated at 4°C, overnight. Secondary antibody was added and the
23 assay was carried out as for the mouse sera. The cut-off was calculated as the mean of the
24 value of the OD obtained with mock cell lysate + 3 SD. The OD obtained for each serum

1 using E2 protein as the solid phase antigen was corrected by subtracting the OD value
2 obtained with the cell lysate expressing β -galactosidase.

3 **b) Inhibition assay.** A decreasing concentration of the competitor mimotope K1 or LCM
4 at 2-fold dilution starting from 100 μ g/ml) was incubated overnight at 4°C with fixed
5 dilution of human sera (1/20). The mixture was then added in duplicate to plates pre-
6 coated with 0.5mg/ml of E2 cell lysate and mock cell-lysate at 4°C, overnight. Each
7 human serum (1/20) without competitor was also added in duplicate to pre-coated plates.
8 The assay was carried out as described above. The OD values obtained with mock-cell
9 lysate were subtracted from OD values obtained with E2-cell lysate. The percentage of
10 inhibition was calculated as the ratio between the net OD value in presence of inhibitors
11 and direct binding of human sera to E2 protein in absence of inhibitor x 100.

12 **c) Competition ELISA.** The assay was performed as in the inhibition assay except that a
13 decreasing concentration of partially purified E2 protein (2 fold dilution, starting from
14 20 μ g/ml) was added in duplicate simultaneously with the synthetic peptide competitor and
15 human sera to plates coated with the same concentration of protein. No mock cell lysates
16 were included in the diluent. The reactants were incubated for three hours. Sera were also
17 added in duplicate to the antigen-coated plates without competitors.

18 Competition was measured as the inhibition exerted by any of the competitors for the
19 binding of anti-E2 containing human sera to the E2-coated ELISA plates, and calculated
20 as a percentage of the direct binding to the E2-coated plates. The assay was done in
21 duplicate on each occasion. The results were calculated as the percentage of binding of the
22 human sera in the presence of the competitor to direct binding to the E2-coated ELISA
23 plates.

24

E2-CD81 Binding Assay

This assay was performed as described by Flint *et al.*, (1999) [Flint *et al.*, 1999]. Briefly, GST-CD81 (EC2) encoding the human (hCD81) and murine (mCD81) second extracellular (EC2) loops were coated onto Immulon II ELISA plates at 5µg/ml in PBS. Plates were blocked with 4% milk powder/TBS. A truncated E2 661 protein (H-Chiron) at 500ng/ml, the concentration resulting in 50% of maximal binding to CD81, was incubated with human serum at final dilutions of 1/200 and 1/1000 at room temperature for 1 hour. The E2-antibody mix was added to the CD81 coated plates in triplicate and allowed to bind at room temperature for 2 hours. After washing with Tris phosphate buffer saline (10mM Tris-HCl pH8.0, 0.15M NaCl), bound E2 was detected with rat mAbs 7/59, 6/1a and 11/20a and anti-rat IgG horseradish peroxidase. Human mAb CB H7 and CB H9 positive and negative controls respectively were included in the assay to assess the validity of the assay.

RESULTS

Selection Of HCV E2 Protein Mimotopes

A peptide mimic of the epitope recognised by mAbs 3e5-1 and 5E5/H7 was identified. The percentage yield after the third round was significantly increased >200 for mAb 3e5-1 and ~500 times for mAb 5E5/H7, than the background library (Table I) giving rise to 15 and 21 clones respectively. All 36 clones showed reactivity when screened by immuno-dot blot to their specific mAb (results are not shown). DNA sequencing of the insert of all enriched phage clones for both mAbs revealed an identical 15 deduced amino acids, F G E F S F Q Y D C S S W G C, named mimotope K1. Analysis using the FASTA computer

1 program [Pearson and Lipman, 1988] showed a lack of similarity between the mimotope
2 K1 with any known human protein.

3 The specificity of the mimotope enriched phage clones for binding to these anti-HCV E2
4 mAbs was confirmed by ELISA. mAbs 3e5-1 and 5E5/H7 gave OD_{492nm} values of 2.17
5 and 0.849, respectively (Figure 1). In contrast, the same antibodies gave OD_{492nm} values
6 of 0.228 and 0.593 when tested against an unrelated phage clone, respectively. These
7 results indicated that the immuno-reactive phage clones were specific to antibody 3e5-1
8 than to 5E5/H7. 5E5/H7 antibody had a substantial cross reactivity with the unrelated
9 phage clone.

11 **Delineation Of HCV Epitope Mimicked By The Mimotope K1**

12 The K1 peptide appears to be a mimic of the peptide ⁵²³GAPTYSWG⁵³⁰ of E2 protein
13 (alternatively we can say: the K1 peptide could substitute the authentic peptide
14 ⁵²³GAPTYSWG⁵³⁰ of E2 protein). This peptide had the greatest binding among nine 7-mer
15 peptides recognised by mAb 5E5/H7 in PepScan analysis [El-Attar et al. in preparation].
16 Alignment of this peptide, the mimotope K1, HCV prototype H strain and other HCV
17 types 1a, 1b, 2a, 2b, 3a, 4, 5a and 6 (Figure 2) revealed the presence of several identical
18 and conserved residues within this region of E2. The sequence/motif S₁S₂WG has the core
19 motif WG identical to all HCV types, whereas S₁ and S₂ was replaced by either identical
20 (S) or conserved residues (S, N, T and Y) with the exception of R in HCV-BK isolate. S,
21 N, T and Y residues are uncharged, polar amino acids. Interestingly the core motif WG
22 has been reported to be critical for binding E2 to CD81 [Owsianka et al., 2006] and the
23 peptide mimicked by K1 overlaps with the sequence defined as CD81 binding region 2
24 [Yagnik et al., 2000].

Immunogenicity And Antigenicity Of Mimotope K1

Mice immunised with chimeric peptide/in FCA induced antibody responses mainly to the mimotope K1. The geometric mean titre to the chimeric and the mimotope K1 peptides were \log_{10} 3.3 and \log_{10} 2.9, respectively.

The murine anti-mimotope antibodies were found to recognise the native E2 protein of HCV. Binding of the anti-mimotope antibodies to E2 protein was confirmed by repeating the experiment 5 times. Antibody titres when using the COS-7 cell lysate infected with RAd-79 as solid phase antigen were significantly higher in sera of immunised mice (Table II) as compared to those from control mice (Table II) (p value was 0.0003 by student t test). Similarly, in three independent experiments, the anti-mimotope antibody titre to partially purified E2 protein was significantly higher (p values of 0.0292, 0.04920 and 0.0089, respectively by student t test) as compared to that of the negative group (Figure 3). To avoid any false evaluation, a *Bonferroni* correction was calculated and p value was equal to 0.0000.

Prevalence Of Anti-E2 Antibody Response In HCV-patient Sera

All 15 (100%) human sera positive (on going infection) or weak positive (self-limited infection) for HCV-RNA had anti-E2 antibodies compared to 7 out of 10 (70%) sera negative to HCV RNA (resolved infection). However, among the 8 sera negative for anti-HCV antibodies, 3 sera (37%) showed the presence of anti-E2 antibodies. This may be due to the low sensitivity of the commercial ELISA tests for screening anti-HCV antibodies resulting from the lack of envelope protein in these assays. It may also indicate the appearance of anti-E2 antibody earlier in the infection, and this could be missed out by a commercial ELISA. These sera were excluded from this study. Antibody titres were

1 generally low among all anti-E2 antibody positive sera detected, ranging between \log_{10} 1.3
2 - 3.1 (results not shown) compared to <1.3 in the negative sera.

3 4 **The Mimotope K1 Inhibits Binding Of Human Anti-E2 Antibodies To Native E2** 5 **Protein.**

6 The binding of 5 out of 7 (71%) sera with a confirmed history of hepatitis C infection to
7 the E2-lysate was inhibited by the K1 mimotope at the concentration of 0.5mg/ml. The
8 inhibition value ranged between 24% - 59% (Figure 4). Four (80%) of these sera were
9 negative or weakly positive for HCV RNA. These results highlight a possible correlation
10 between recovery from HCV infection and the presence of anti-E2 antibodies.

11 One sample, which had shown no reactivity, was initially anti-E2 antibody negative. This
12 particular serum was chosen to ensure the sensitivity of the assay. No reaction was
13 detected with the negative sera in presence or absence of the mimotope K1. One serum
14 with antibodies to the E2 lysate was inhibited by 18% with the unrelated peptide.

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16 To emphasise the value of the mimotope in terms of resolving infection for use with
17 human sera, a further competition assay was performed with nine HCV sera from
18 individuals with self limited or resolved hepatitis C infection. The mimotope K1 was able
19 to compete with the recombinant E2 protein in four out of 6 cases where E2 had a degree
20 of inhibition. In three cases the inhibition induced by the mimotope was 18.3 %, 38.12 %
21 and 24.7% compared to 2.75 %, 8.04 % and 28.62% with recombinant E2 protein
22 respectively (Figure 5). In the fourth case the inhibition exerted by the recombinant E2
23 protein was much greater than seen using the mimotope where the percentage of
24 inhibition was 31.3% and 13.9% respectively. The titre of anti-E2 protein antibody ranged

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5 1 between \log_{10} 1.9 - \log_{10} 2.2 in these four sera. Some inhibition was observed with the
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7 2 control peptide in two patients (15.61% and 19.11%).
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4 **Recognition Of The Mimotope K1 By HCV Human Sera With The Capacity To** 5 6 **Block E2 Binding To CD81.**

7 To assess the value of the mimotope in terms of recognition by human sera that are
8
9 7 capable of inhibiting E2- CD81 interaction, the nine sera used for competition assay were
10
11 8 tested for their ability to block E2 binding to CD81. Five sera had inhibited E2 binding to
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13 9 CD81 at a 1/1000 dilution. Four of these sera were found to be reactive with the
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15 10 K1mimotope (Table III), suggesting that the mimotope could be a mimic of an epitope on
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17 11 E2 protein that is involved in E2-CD81 interaction. The human mAb CBH-7 showed
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19 12 inhibition activity as expected and negative control CBH-9 mAb had no serum inhibition
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21 13 activity.
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38 **DISCUSSION**

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43 17 There is accumulating evidence that CD81 is playing a central role in HCV entry to
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45 18 hepatocytes through its interaction with E2 protein. Identification of an inhibitor to block
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47 19 this interaction would assist future therapeutic vaccine to control HCV infection.
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51 20 In this study a mimotope was identified that mimics a conformational B-cell epitope
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53 21 involved in binding of E2 to CD81 using 15-mer random phage displayed peptide library.
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55 22 The mimotope was able to elicit HCV E2 specific antibody responses *in vivo* and bound to
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57 23 human sera with the capacity to inhibit E2-CD81 interaction. Two murine mAbs, 3e5-1
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59 24 and 5E5/H7, raised against the E2 protein were selected for screening. mAb 3e5-1 that
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1 recognises a linear epitope and mAb 5E5/H7 that recognises a conformational epitope
2 [Houghton M, personal communication]. mAb 5E5/H7 was of more interest as it was
3 initially used to purify E1-E2 heterodimer complexes used in the chimpanzee protection
4 study [Choo et al., 1994; Rosa et al., 1996], highlighting that an epitope recognised by
5 mAb 5E5/H7 would be a mimic of the authentic protein on the virus particle. mAb
6 5E5/H7 gave significantly higher yields of reactive clones after three rounds of
7 biopanning. The reactivity of each mAb for the selected clones was confirmed by
8 immuno-blot and ELISA, with mAb 3e5-1 giving the best discrimination between selected
9 phage clones and unrelated phage clones. This could be explained by the linear nature of
10 the epitope recognised by mAb 3e5-1 and the complex nature of the epitope recognised by
11 mAb 5E5/H7. This is supported by previous characterisation of these two monoclonal
12 antibodies (Michael Houghton Invention EP0980434B1
13 (<http://www.freepatentsonline.com/EP0980434.html>)) where evaluation of epitope
14 exposure by both antibodies showed mAb 3e5-1 giving approximately 3.9 higher signal to
15 intracellularly E2 compared to conformational mAb 5E5/H7.

16
17 The conformational nature of the epitope recognised by mAb was initially assessed by
18 PepScan analysis (El-Attar et al, in preparation). PepScan revealed 9 reactive but distantly
19 located E2-overlapping peptides with the peptide ⁵²³GAPTYSWG⁵³⁰ giving the highest
20 ELISA signal. The pattern of reactivity of mAb 5E5/H7 with distantly located peptides
21 suggested that these peptides are likely closely juxtaposed on the surface of the native E2
22 protein confirming the conformational dependant nature of this mAb 5E5/H7. Visual
23 inspection of the mimotope K1 showed possible contact residues within the domain
24 represented by the peptide ⁵²³GAPTYSWG⁵³⁰ namely SWG. The motif is common among
25 several HCV-1 isolates with the core motif WG identical in all HCV types including HCV

1 1a and 1b, the most divergent isolates and flanked by conserved residues, highlighting that
2 the mimotope K1 mimics a conserved domain among different HCV strains. Additionally,
3 the epitope mimicked by the peptide K1 overlaps with a region of E2 protein spanning a.a.
4 522- 535, that is rich in turns and with an antigenicity index inclusive of a substantial
5 amount of β and coil regions (El-Attar et al, in preparation). The presence of tryptophan
6 (W) in the SWG motif is in agreement with the importance of aromatic amino acids in the
7 antigenic determinants of peptides [Appel et al., 1990].

8
9 Interestingly, the predicted contribution to the epitope mimicked by K1 is in agreement
10 with the recent identification of critical conserved amino acid residues involved in binding
11 E2 to CD81 [Johansson et al., 2007; Owsianka et al., 2006] and overlaps with CD 81
12 binding region 2 (a.a 522-551) proposed by Yagnik et al. [2000].

13 Similar regions of E2 of conformational nature (a.a. 480- 493 and 544-551) involved in
14 the CD81 interaction was also reported [Flint et al., 1999]. Other investigators have
15 reported the existence of discontinuous B cell epitope that overlaps with the domain
16 mimicked by K1 [Lechner et al., 1998; Mink et al., 1994]. In one report, rabbit antibodies
17 recognised a domain spanning residues 511-530 of the E2 protein was found to block viral
18 attachment [Lechner et al., 1998]. Studies with human mAbs have revealed the presence
19 of conserved conformational-dependent epitopes [Allander et al., 2000; Bugli et al., 2001;
20 Hadlock et al., 2000; Johansson et al., 2007]. Antibodies to these epitopes inhibited the
21 binding of E2 to CD81.

22
23 To verify that the mimotope K1 is a mimic of authentic HCV E2, anti-mimotope
24 antibodies were raised in mice and tested for their binding to the native E2 protein. In
25 order to overcome immune unresponsiveness due to the low molecular weight of the

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5 1 mimotope K1 the T-helper epitope (288-302) of the fusion protein of measles virus was
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7 2 colinearly coupled to the N terminal of the mimotope K1 peptide. This T-helper epitope is
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9 3 known to have a wide range of class II major histocompatibility complex molecules both
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11 4 in mice and human [Partidos and Steward, 1990].
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14 5 The antibody response induced following immunisation with the chimeric peptide was
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16 6 mainly against the mimotope K1. The level of anti-mimotope antibody titre to the E2 was
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18 7 significant in four out of five experiments (*p* values 0.0003-0.0492). These data
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20 8 demonstrated that the mimotope K1 could substitute for the authentic HCV E2 epitopes in
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22 9 provoking a specific immune response to the E2 protein. The low level of anti-E2 antibody
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24 10 responses obtained in these experiments can be explained by the requirement of
25
26 11 considerable molar excess (10^3 - 10^4) of peptide over native protein in order to reveal any
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28 12 binding between peptide and antibody. Additionally, the affinity constant of the antibody
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30 13 for the peptide is mostly several orders of magnitude lower than for the protein [Van
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32 14 Regenmortel, 1987].
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40 16 The fact that the mimotope K1 mimics the CD81 binding region 2 suggests that anti-
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42 17 mimotope antibodies would have the ability to block binding E2 protein to CD81.
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44 18 However it was not feasible in the course of this study to measure the inhibition capacity
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46 19 of E2-CD81 interaction and Nt activity in anti-mimotope mice sera due to the low anti-E2
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48 20 antibody titre and the limited volume of available mouse sera.
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51 21 Human antibodies elicited in response to HCV infection are anticipated to react with
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53 22 native conformation of the viral envelope structure. We evaluated the mimotope K1 in
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55 23 terms of recognition by a panel of human sera with various HCV profiles and examined its
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57 24 capacity to inhibit their binding to the native protein. These sera were first screened for the
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59 25 presence of anti-E2 antibodies. The level of anti-E2 antibody was generally low in
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1 agreement with previous reports. The mimotope inhibited the binding of all sera that were
2 either negative or weak for HCV RNA compared to 1 of 2 patients with circulating HCV
3 RNA. The specificity of the assay was confirmed as negative human sera as well as HCV
4 patients with no anti-E2 antibodies gave negative results, with no difference in the binding
5 of antibodies to the native protein in the presence or absence of the mimotope. These
6 results add to the value of the mimotope as representing a conformational epitope, which
7 may play a role in viral clearance. In this context, other studies [Keck et al., 2004; Lechner
8 et al., 1998] suggested that a substantial portion of anti-E2 antibodies in HCV infected
9 patients are directed to non-linear B cell epitopes of the HCV envelope protein. It was
10 also demonstrated in the previous study that the K1 mimotope mimics an epitope
11 recognised by convalescent human sera. These sera were examined in this study for their
12 capacity to inhibit E2-CD81 interaction. Five sera inhibited E2-CD81 interaction at 1:
13 1000 dilutions. Four of these sera were reactive with the mimotope by the competition
14 assay. Binding of HCV to cellular ligand e.g. CD81 may be mediated via a domain on E2
15 that overlaps with that defined by the synthetic mimotope K1. Residues 522-551 of E2
16 protein were also reported to be involved in binding to CD81 [Flint et al., 1999; Zhang et
17 al., 2004]. Within the latter region of E2, five residues (W420, Y527, W529, G530 and
18 D535) were critical for CD81 binding [Owsianka et al., 2006], three of these residues are
19 present in the peptide reported in this study giving the highest signal with mAb 5E5/H7
20 and mimicked by the mimotope K1.

21 Antibodies to E2 are found in over 90% of persistently infected individuals but resolution
22 may require antibody against selected epitopes. This antibody specificity may be absent in
23 chronic hepatitis C disease. Such a gap in the antibody repertoire has been suggested as
24 occurring in carriers of HBV [Brown et al., 1984]. Notwithstanding this, the development
25 of peptide mimics of conformational E2 determinants could prove valuable for the fine

1 analysis of antibody responses against this protein, possibly taking into account the role of
2 protein and adjacent E1 sequences in determining the correct folding of E2.

3 The mimotope K1 mimics an epitope that lies in an area that displays a high degree of
4 homology within HCV subtypes (92-97% identity) indicating that antibodies directed to
5 this mimotope would cross-react with different HCV isolates. The reactivity of two
6 broadly neutralising human monoclonal antibodies was reported to be dependent on
7 residues in this same region of E2 (amino acids 523-535) [Johansson et al., 2007].

8
9 In summary, the mimotope K1 was able to induce HCV E2 specific immune response *in*
10 *vivo* in mice and binds to human sera from patients infected with HCV which showed the
11 capacity to inhibit E2-CD81 interaction. This response was directed at a conformational B
12 cell epitope that displays a high degree of homology within HCV subtypes (92-97%
13 identity), thus would broadly cross-react with different HCV isolates. More work is
14 needed to enhance the anti-mimotope antibody response and to assess the ability of
15 mimotope K1 for the induction of neutralising antibody responses. In conclusion, the
16 results of this study offer an approach for the development of therapeutic vaccines against
17 hepatitis C.

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20
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For Peer Review

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