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Towards a solution for hepatitis C virus hypervariability: mimotopes of the hypervariable region 1 can induce antibodies cross-reacting with a large number of viral variants

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The hypervariable region 1 (HVR1) of the putative envelope protein E2 of hepatitis C virus (HCV) is the most variable antigenic fragment in the whole viral genome and is mainly responsible for the large inter- and intra-individual heterogeneity of the infecting virus. It contains a principal neutralization epitope and has been proposed as the major player in the mechanism of escape from host immune response. Since anti-HVR1 antibodies are the only species shown to possess protective activity up to date, developing an effective prevention therapy is a very difficult task. We have approached the problem of HVR1 variability by deriving a consensus profile from >200 HVR1 sequences from different viral isolates and used it as a template to generate a vast repertoire of synthetic HVR1 surrogates displayed on M13 bacteriophage. This library was affinity selected using many different sera from infected patients. Phages were identified which react very frequently with patients' sera and bind serum antibodies that cross-react with a large panel of HVR1 peptides derived from natural HCV variants. When injected into experimental animals, the 'mimotopes' with the highest cross-reactivity induced antibodies which recognized the same panel of natural HVR1 variants. In these mimotopes we identified a sequence pattern responsible for the observed cross-reactivity. These data may hold the key for future development of a prophylactic vaccine against HCV.

Keywords: hepatitis C virus/hypervariable region 1/ phage library

Introduction

Hepatitis C virus (HCV) is the major etiological agent of both blood-transfusion-associated and sporadic non-A non-B hepatitis worldwide, with an estimated prevalence of 0.4–2% in the blood donor population (Choo *et al*., 1989). HCV infection leads to viral persistence and chronic disease in at least 70% of cases, among which a significant proportion eventually develops cirrhosis and hepatocellular carcinoma (for a review see Alter, 1995). In spite of the availability of reliable serological tests for HCV diagnosis, community-acquired infection is still common and causes significant morbidity and mortality worldwide (Mast and Alter, 1993). In addition, interferon treatment, which is the only anti-viral therapy available at the moment, is effective in only 20–30% of patients (Fried and Hoofnagle, 1995). Thus, development of an HCV vaccine is a high priority project.

The high frequency with which the virus establishes a persistent infection, despite a wide array of humoral and cell-mediated host immune responses, raised some concern about the existence of a protective immunity against HCV in the past (Farci *et al*., 1992). Protective immunity against challenge with homologous virus has since been induced by vaccination of chimpanzees (the only other species susceptible to HCV infection) using recombinant forms of the putative envelope proteins E1 and E2 (Choo *et al*., 1994). However, it remains to be established how effective this response would be against heterologous viral inocula. In fact, HCV exists in the bloodstream of infected patients as a quasispecies (Weiner *et al*., 1991; Martell *et al*., 1992, 1994; Kurosaki *et al*., 1994; Bukh *et al*., 1995) which fluctuates during the course of the disease mainly as a result of immune pressure (Weiner *et al*., 1992, 1995; Kato *et al*., 1993; Kojima *et al*., 1994; Shimizu *et al*., 1994; van Doorn *et al*., 1995). The emerging view is that chronic infection by HCV is not due to lack of humoral or cellular responses, but rather to their being rendered ineffective by the high mutation rate of the virus which produces escape variants.

The existence of neutralizing antibodies and their role in protection from viral infection was documented by *ex vivo* neutralization of a pedigreed viral inoculum prior to injection into chimpanzees (Farci *et al*., 1994). However, these antibodies were proved to be isolate-specific and seemed able to block only viral variants present before the onset of the corresponding humoral response (Farci *et al*., 1994). Even if their specificity was not well defined, both immunological and molecular evidence indicates that epitopes recognized by neutralizing antibodies are localized in the hypervariable region 1 (HVR1) of the HCV genome (Farci *et al*., 1994). This consists of the N-terminal 27 amino acids of the E2 glycoprotein, the most variable region of the whole HCV polyprotein (Weiner *et al*., 1991). Direct proof of the role played by anti-HVR1 antibodies in virus clearance came recently from *ex vivo* neutralization experiments. A rabbit anti-HVR1 hyperimmune serum raised against the predominant variant of an infectious HCV inoculum abolished its infectivity in one chimp, and partially protected a second animal by blocking propagation of the major variant present in the inoculum (Farci *et al*., 1996).

Thus, it appears that the HVR1 contains a principal

neutralization determinant for HCV, which would constitute an essential component of an acellular HCV vaccine, if the problem of its variability could be surmounted. Anti-HVR1 antibodies from human sera display some degree of cross-reactivity to different HVR1 variants (Scarselli *et al*., 1995). On close inspection of this region we observed that some positions of the HVR1 are less variable than others (this work), suggesting that the structural and immunological variability is more limited than the heterogeneity found in the primary sequence. If this hypothesis is correct, one or more 'synthetic variants' of the HCV HVR1 could conceivably be generated which are immunologically similar to a great number of natural HVR1 variants and therefore able to induce neutralizing antibodies which cross-react with most or all HCV variants.

Phage-displayed peptide libraries offer the unique opportunity of rapidly surveying large collections of peptide sequences (10^8 or more) through a cyclic selection/rescue/amplification procedure. They allow ligands to be identified for any type of ligate ranging from linear peptides to folded protein domains, and even carbohydrates (Cortese *et al*., 1994, 1996). These ligands are true mimotopes as they do not necessarily share the same amino acid sequence of the original epitope, but they mimic its binding properties. We have previously reported a strategy for the identification of disease-specific phage-displayed mimotopes which avails itself only of clinically characterized sera from immune and non-immune individuals (Folgori *et al*., 1994). Disease-specific mimotopes also proved efficient immunogenic mimics of the natural antigen, as they induced a specific immune response to it when injected into different animals (Folgori *et al*., 1994; Meola *et al*., 1995; Mecchia *et al*., 1996; Prezzi *et al*., 1996). Thus, phage libraries can be used as a source of artificial ligands recognized by disease-specific antibodies, with the advantage that additional desirable features can be built in, providing they can be selected for during library enrichment.

We have approached the issue of HVR1 variability by generating a vast repertoire of HVR1 surrogates displayed on bacteriophage M13 as fusion to the major coat protein (pVIII). Using the power of selection and many sera from clinically characterized HCV-infected individuals, we have isolated peptides which have been proved to be effective antigenic and immunogenic mimics of a large number of naturally occurring HCV variants.

Results

Design and construction of a specialized phage library mimicking the HVR1 variability

We used a multiple sequence alignment of 234 unique HVR1 sequences extracted from sequence databases to characterize the variation in residue composition at each of the 27 N-terminal positions of the HCV E2 glycoprotein (Materials and methods). A sequence pattern emerged from this analysis (Figure 1A), allowing the definition of a degenerate consensus sequence. The conservation pattern observed in this consensus could be responsible for the observed cross-reactivity of human sera to different HVR1 variants (Scarselli *et al*., 1995). Based on this hypothesis we decided to design a synthetic repertoire of HVR1 sequences which would contain these conserved constraints while reproducing the natural variability observed in the remaining positions.

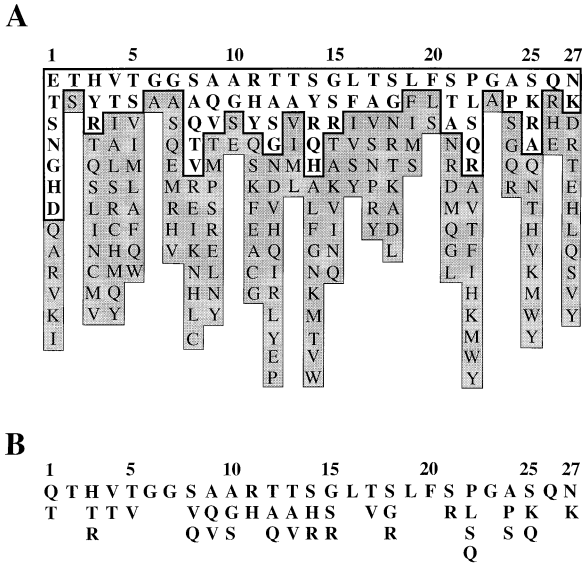

Fig. 1. Derivation of the HVR1 library consensus profile. (**A**) Consensus pattern of the 234 natural variants of the HCV HVR1 sequences used in this work. Non-shaded residues within the box account alone for ~80% of the observed variability. Residues are listed in decreasing order of observed frequency from top to bottom. (**B**) HVR1 library composition.

We derived a consensus-profile that accounted for ~80% of the total sequence variability by selecting the most frequent residues at each position. When similar amino acids were present at a given position, only one was chosen to represent variability. For example, in position 5 both Ser and Thr are present in the natural repertoire, but only Thr was selected to design the library as the total number of sequences needed to be kept within the upper practical limit (~10^8) of current DNA cloning and transformation techniques (Figure 1). In some cases, a residue not present in the consensus was included to better mirror the overall variability, for example Thr was included in position 3 to account for polar residues present in the natural repertoire of HVR1s.

The resulting final consensus profile (Figure 1B) has a complexity of 9×10^7. The amino acid most frequently observed in the natural repertoire was always included, with the exception of position 1, where Gln and Thr were selected. Eight positions (2, 6, 7, 16, 19, 20, 23 and 26) were kept constant, given the high local sequence conservation throughout the 234 natural HVR1 variants. Qualitatively, the profile can be described as a generally more variable central region flanked by N-terminal and C-terminal tails containing conserved elements.

Construction of the library proceeded by cloning a degenerated synthetic oligonucleotide as a fusion to the 5′ end of the gene coding for the major coat protein (pVIII) in a phagemid vector for M13 display (see Materials and methods). Approximately 2×10^8 independent transformants were obtained. To verify the quality and complexity of the HVR1 library we sequenced the inserts of 56 randomly chosen individual clones with the following results: (i) all clones displayed different sequences; (ii) 63% of the clones contained full-length inserts while the

Table I. Scheme of the selections

Ist selection serum/genotype	phage pool	IInd selection serum/genotype	phage pool
σ4R (1b)	4R	σ2 (1b)	B
σ3R (3a)	3R	σ1 (1a)	D
σ3 (2a)	3	σ2 (1b)	E
σ2R (3a)	2R	σ3 (2a)	R
σ1 (1a)	1	σ4 (2a)	F
		σ2 (1b)	H
σ2 (2b)	2P	σ2 (1b)	G
		σ1 (1a)	L
		σ4 (2a)	M
σN	N		

First and second round of HVR1 library enrichment with sera from HCV-infected patients are indicated at the top of the table. Names of the sera and genotype of the corresponding infecting virus (in parentheses) are shown in the left column. Indicated in the right column are the names of the resulting phage pools.

remainder had small deletions; (iii) none of the sequenced clones encoded for peptides corresponding to known HVR1 from viral isolates (data not shown). From these data we inferred that the library has a complexity close to the number of individual transformants.

Identification of HVR1 mimotopes that frequently react with HCV patients' sera

The more complex and diverse the repertoire of antibodies used for selection, the higher the probability of enriching phage recognized by many different antibodies against HVR1 epitopes. Sera from chronically infected, viremic patients would appear to possess this highly heterogeneous population of anti-HVR1 antibodies, as these individuals have a rather long history of viral persistence, during which a large number of HCV variants have been generated and have challenged the immune system.

We used eight sera from chronic patients infected by viruses of five different genotypes, 1a, 1b, 2a, 2b and 3a (Simmonds *et al*., 1993), to perform six affinity selections of the HVR1 library (Table I). As a control, a serum from a non-infected individual was also used. Pools of phage obtained from all seven selections were amplified and tested for their reactivity to each of the selecting sera in ELISA. The results of this experiment showed a significant enrichment of phage recognized by the selector antibodies, as demonstrated by the increase in reactivity with respect to the unselected library (Figure 2). Peptides recognized by antibodies unrelated to HCV infection were also enriched from the library. In fact, the pool of phage selected with the control serum has a higher reactivity with this serum than the unselected library (Figure 2). However, patients' sera drove selection toward HCV-related mimotopes as no reactivity to phage pools enriched by HCV sera was detected using sera from healthy individuals (Figure 2 and data not shown).

In most cases, phage pools enriched by HCV sera reacted with more than one patient's serum. To gain insight into the reactivity frequency of the selected mimotopes with different patients' sera, 40 individual clones from two pools (4R and 2R, Table I) were randomly chosen and tested for their reactivity in ELISA with a panel of 20 sera, different from those used for the selection, from HCV infected patients. An equivalent number of sera from non-infected healthy controls were used to assess the specificity for anti-HCV antibodies. Twenty-four clones turned out to be HCV-specific, and their distribution as a function of their frequency of reactivity with patients' sera is reported in Figure 3 (upper panel). Among them, phage reacting with more than one serum (up to 55% of the tested samples) were identified.

To further improve the isolation of mimotopes reacting with many different anti-HVR1 antibodies, we subjected the enriched phage pools to a second round of affinity selection using patients' sera different from those used for the first round. In this way nine new pools were generated (Table I) and analyzed by ELISA. As before, a general increase in reactivity with the selector antibodies was observed. In addition, all second round phage pools reacted more frequently than those selected in the first round with a panel of sera from HCV-infected patients different from those used for either selection (data not shown), reflecting a higher recognition frequency of the isolated peptides. This was confirmed by comparing the frequency of reactivity with HCV sera of clones from the first and second round of affinity selection (Figure 3). The distribution of reactivity also appeared significantly different after the second selection step; clones isolated after two rounds of selection show a bell-shaped distribution as opposed to scattered distribution of phage from the first selection (Figure 3), indicating that the whole phage population had indeed acquired more of the desired binding properties. We decided to omit additional selection cycles to avoid introduction of a bias toward biologically favoured phage during amplification.

A total of 200 clones reacting exclusively with HCV sera were identified by screening all second-round pools. Their distribution as a function of the recognition frequency by HCV sera mirrored that of the subset displayed in Figure 3, with the best clones reacting with 80% of the tested samples (data not shown). More importantly, despite their quantitative similar overall frequency of recognition by the HCV sera, different clones display a characteristic pattern of reactivity with the net result that few mimotopes score for the presence of anti-HVR1 antibodies in all tested sera (Figure 4A).

We next verified whether the observed high frequency of recognition by HCV sera was limited to the tested patient population or whether it reflected an intrinsic property of the selected mimotopes. For this purpose another set of sera from infected patients was assayed by ELISA, revealing that both the frequency of reactivity of each individual phage and the total coverage of the sera remained unaltered (Figure 4B).

HCV patients who have resolved infection have most likely come in contact with a lower number of viral variants and presumably developed a narrower spectrum of variant-specific anti-HVR1 antibodies than chronically infected patients. This hypothesis is supported by the finding that sera from the former population react with synthetic peptides reproducing the HVR1 of natural isolates much less frequently than those of chronically infected viremic patients (Scarselli *et al*., 1995; data not shown). Therefore, non-viremic sera could constitute a better and more stringent test for assaying the cross-reactivity of HVR1 mimotopes with different anti-HVR1 antibodies.

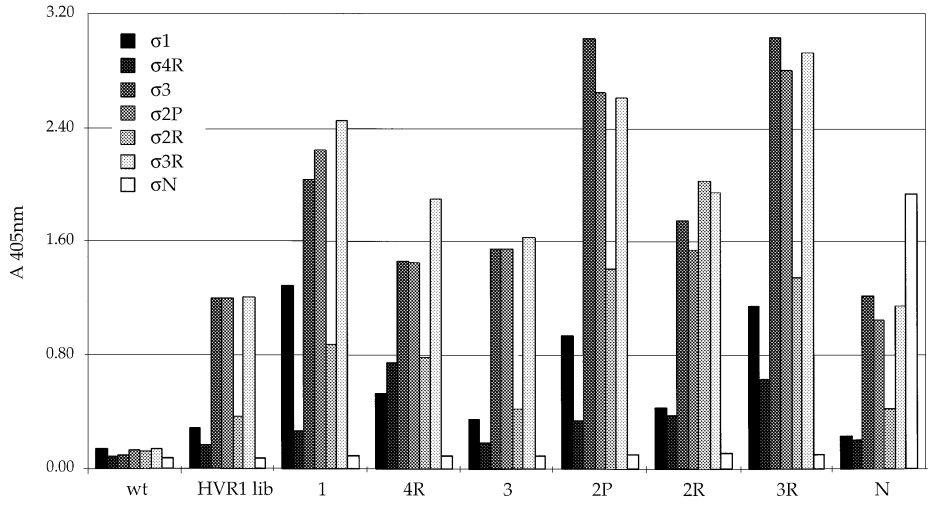

Fig. 2. Reactivity of phage pools yielded by the first round of affinity selection to IgG present in the selecting sera. Binding of the selected phage pools to the tested antibodies was detected by ELISA on immobilized phage as described in Materials and methods. For each serum sample, indicated at the top left corner of the figure, antibody recognition of the phage pools (1, 4R, 3, 2P, 2R, 3R and N), wild-type phage (wt) and the unselected library (HVR1 lib) was measured. Average values (A_{405}) from two independent experiments have been determined.

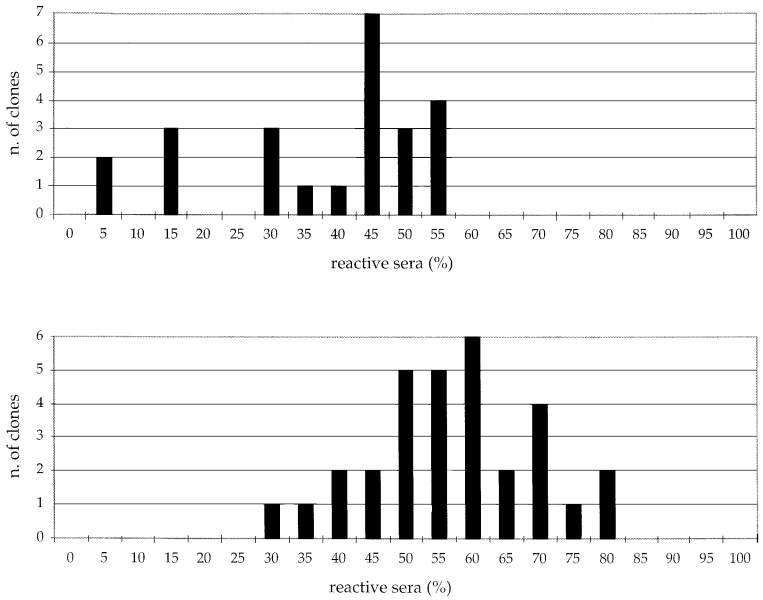

Fig. 3. Distribution of HCV-specific phage selected from the HVR1 library as a function of their frequency of reactivity with sera from infected patients. Binding of phage enriched by one (top panel) or two (bottom panel) cycles of affinity selection to antibodies present in 20 human sera different from those used for selection was detected by ELISA on immobilized phage as described in Materials and methods. For each serum, average values (A_{405}) from two independent experiments have been determined on the selected phage and on wild-type phage. Values were considered statistically significant when differing $>3\sigma_{max}$ ($P < 0.003$) from the background signal observed for the wild-type phage. Each histogram represents the number of phage clones (shown on the vertical axis) that react with the indicated number of sera expressed as percentage of total number of tested samples (horizontal axis).

Some of the selected mimotopes were thus tested against 41 samples from HCV-seropositive individuals who were repeatedly found to be negative for the presence of viral RNA in the blood. Again the mimotopes reacted with many of these sera, albeit at a lower frequency than that observed with sera from viremic patients (compare A, B and C in Figure 4). We interpret these data as an indication of the ability of the selected mimotopes to cross-react with a large number of different anti-HVR1 antibodies.

Relationship between the sequence of the selected HVR1 mimotopes and their frequency of reactivity with HCV sera

We wanted to verify whether the amino acid sequence of the selected clones is correlated with the frequency of reactivity. No obvious pattern arises from a visual comparison of the sequences, so we decided to analyze separately the sequence patterns of the least and most frequently reacting clones.

A

	R9	H1	M63	M122
C7	0.14	0.13	0.00	0.22
C8	1.67	1.83	1.05	0.38
C9	2.30	0.65	0.21	0.11
C10	1.23	1.30	0.71	0.64
C12	0.16	1.26	0.20	1.09
C13	0.90	2.06	0.57	0.19
C14	0.47	0.63	1.09	2.27
C17	1.48	1.35	1.00	0.00
C23	0.00	0.88	2.65	0.00
C27	0.00	0.00	0.00	3.17
C28	2.98	0.15	0.00	1.98
C29	0.00	0.00	2.85	1.17
C30	0.18	0.00	2.85	1.17
C40	3.17	1.84	0.20	2.96
C48	3.07	2.58	0.00	2.56
C49	0.12	0.00	0.00	0.00
C50	0.00	0.00	0.12	0.00
C56	0.33	0.12	0.00	0.00
C58	2.85	1.42	0.38	0.35
C62	0.00	0.00	0.13	0.00
				total:
frequency	75%	70%	70%	70%	100%

B

	R9	H1	M63	M122
1V	0.49	2.51	0.00	0.20
2V	0.00	0.19	0.11	0.00
3V	0.65	1.90	1.73	0.62
4V	0.25	0.00	0.00	0.29
5V	1.75	2.47	0.31	0.00
7V	0.60	1.09	1.16	0.88
8V	0.30	0.88	0.00	0.00
9V	0.00	0.59	0.00	0.00
10V	0.58	0.11	1.98	0.00
1P	0.00	0.00	0.00	0.33
5P	0.24	0.28	0.44	0.00
6P	0.47	1.54	0.27	0.31
7P	0.78	1.33	1.00	0.00
8P	0.68	0.57	0.14	0.38
10P	1.20	1.12	0.81	0.88
11P	0.32	0.44	0.00	0.22
12P	1.50	1.08	0.84	0.90
13P	0.49	0.00	0.00	0.31
				total:
frequency	83%	83%	61%	61%	100%

C

	R9	H1	M63	M122
X1	0.00	0.00	0.00	0.00
X2	1.37	0.17	0.21	0.68
X3	0.15	0.56	0.38	0.00
X4	0.00	0.00	0.00	0.00
X5	0.00	0.14	0.00	0.00
X6	0.25	0.00	0.00	0.31
X7	0.66	0.83	0.60	0.34
X8	1.04	1.31	0.98	0.45
X9	0.96	0.21	0.00	0.00
X10	0.00	0.00	0.00	0.00
X11	0.74	0.33	0.11	0.91
X12	0.00	0.00	0.00	0.00
X13	2.30	0.18	0.54	0.00
X14	1.20	0.00	0.00	0.00
X15	2.38	0.00	0.00	1.60
X16	1.92	2.30	1.86	2.19
X17	0.00	0.00	0.00	0.00
X18	0.00	0.00	0.00	0.00
X19	0.00	0.00	0.00	0.00
X20	0.47	0.33	0.26	0.00
X21	0.00	0.86	1.45	1.96
X22	0.37	0.44	0.44	0.12
X23	0.61	0.60	0.10	0.00
X24	0.00	0.00	0.80	0.73
X25	0.00	0.00	0.00	0.47
X26	0.00	0.00	0.00	0.00
X27	0.00	0.00	0.00	0.00
1NV	0.00	1.50	0.22	0.00
2NV	0.00	0.40	0.23	0.00
3NV	0.10	0.11	0.00	0.00
4NV	0.00	0.00	0.00	0.00
5NV	0.00	0.00	0.00	0.00
6NV	0.00	0.00	0.00	0.00
11NV	0.10	0.00	0.00	0.53
12NV	0.00	0.00	0.41	0.00
13NV	0.00	0.00	0.00	0.00
15NV	0.12	0.00	0.00	0.19
16NV	0.52	0.84	0.36	0.60
17NV	0.64	0.52	0.41	1.14
3P	0.00	0.00	0.00	0.00
4P	0.00	0.00	0.00	0.00
					total:
frequency	46%	44%	41%	37%	63%

Fig. 4. The selected mimotopes are frequently recognized by antibodies present in human sera from HCV infected patients. Binding was detected by ELISA on immobilized phage. (**A**) Reactivity of mimotopes with the panel of twenty HCV patients' sera used for screening. (**B**) Reactivity with an additional panel of sera from HCV-infected viremic patients. (**C**) Reactivity with sera from non-viremic patients that were scored positive for anti-HCV antibodies using commercially available kits (see Materials and methods). Mimotope names are indicated at the top of each column. For each serum (indicated on the left of each row), average values (A_{405}) from two independent experiments have been determined. Results are expressed as the difference between the average value of the tested phage clone and that of wild-type phage. Positive values are indicated in bold. We considered values as statistically significant when differing $>3\sigma_{max}$ ($P < 0.003$) from the background signal observed for the wild-type phage. The frequency of reactivity of each mimotope and the sum of reactivities observed with all four mimotopes are shown at the bottom of each panel.

We defined as 'weak' the 25 clones that reacted with <3 sera and 'strong' the 29 reacting with >11 sera. Their consensus sequences are shown in Figure 5A, and the amino acid frequencies at each position of weak and strong clones are listed in Materials and methods.

There is a clear trend for some positions to be occupied by different amino acids in the sets of weak and strong clones and this allowed us to define heuristically a position-based scoring system described in Materials and methods. The higher the score of a clone (S-score), the closer its sequence to those of the strong clones and the more diverse from those of the weak ones. As shown in Figure 5B, the S-score correlates reasonably well (correlation coefficient = 0.79) with the experimentally determined frequency of reactivity of each clone. It should be emphasized that the S-score was calculated using only the sequences of the weak and strong clones (54 out of 180), but it correlates well with the frequency of reactivity of all

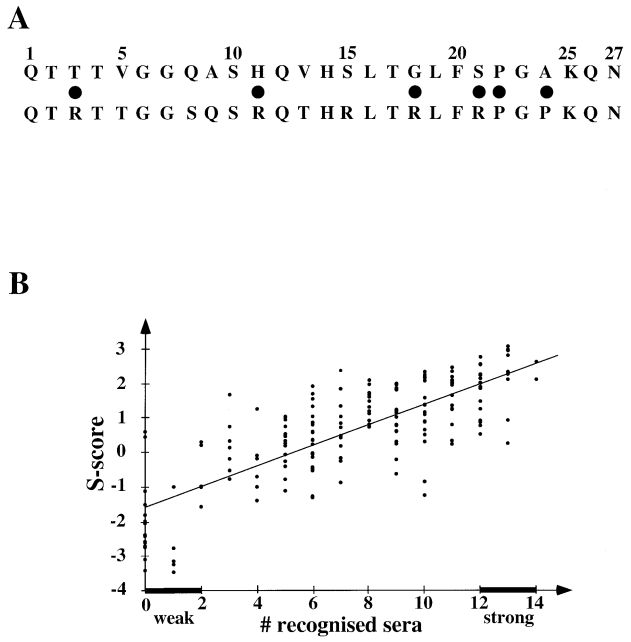

Fig. 5. Mimotopes frequently reacting with patients' sera show a specific consensus. (**A**) Consensus sequence of strong (upper line) and weak (lower line) clones. Positions showing significant differences in amino acid frequencies between the two sets of clones are indicated with a black dot. For example, proline is the most represented amino acid at position 22 in both sets of sequences, but its frequency distribution is significantly different (see Materials and methods). (**B**) Correlation between the S-score and the frequency of reactivity of the selected mimotopes. The S-score has been calculated as described in Materials and methods. The straight line represents the linear least square fit of the data. The correlation coefficient is 0.79.

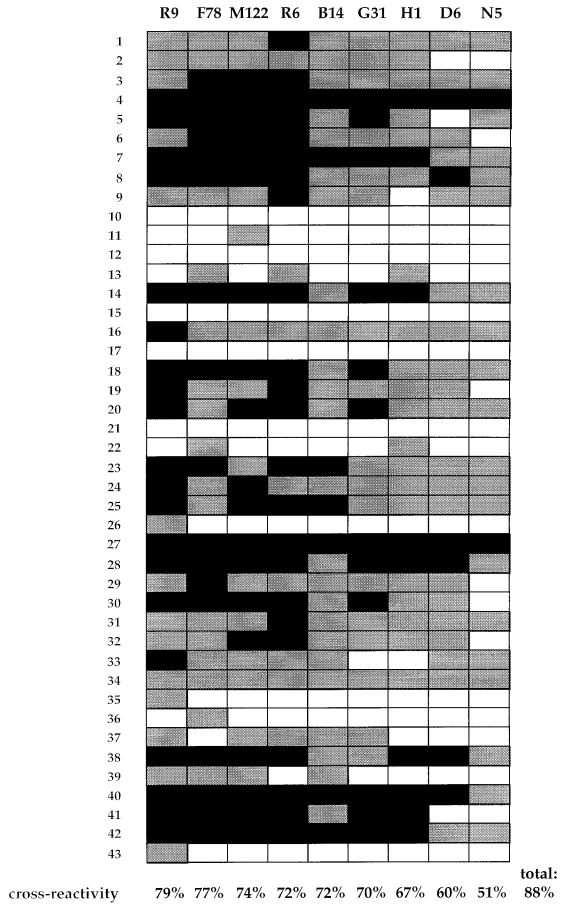

Fig. 6. The selected mimotopes are antigenic mimics of a large number of natural HVR1 variants. Antibodies from a pool of HCV infected sera were immunopurified on MAPs reproducing the sequence of selected mimotopes (top of the figure). Reactivity of equal amounts of immunopurified antibodies was measured by ELISA on a panel of HVR1 sequences synthesized as MAPs (left column; see Materials and methods). Average values from two independent experiments were determined. Values were considered statistically significant when two criteria were simultaneously fulfilled: (i) values differed $>3\sigma_{max}$ ($P < 0.003$) from the background signal observed on two unrelated peptides; (ii) values differed $>3\sigma_{max}$ ($P < 0.003$) from the average signal observed using 10 sera from non-infected individuals on each HVR1 peptide. Grey boxes indicate values differing from those observed on the unrelated MAPs between 0.15 and 0.5 OD (405 nm); black boxes indicate values differing >0.5 OD (405 nm). The level of cross-reactivity of each pool of immunopurified antibodies is indicated at the bottom of each column.

clones. Interestingly, a nearly identical result (correlation coefficient = 0.75) was obtained using only six positions where the residue preference of the weak and strong mimotopes differ most (positions 3, 11, 18, 21, 22 and 24 in Figure 5A). The C-terminal part of HVR1, where four of the six positions are located, could play a particular role in determining the frequency of reactivity. These data suggest that the HVR1 region, termed 'hypervariable', is constrained in its variation, and shows a degenerate consensus that could explain why it is recognized by many antibodies present in different patients' sera.

HVR1 mimotopes antigenically mimic a large number of HVR1 variants from HCV isolates

If the selected HVR1 mimotopes are good antigenic mimics of many different HVR1 natural variants, they should contain elements that fit the paratopes of many different anti-HVR1 antibodies. To verify this hypothesis we measured the cross-reactivity to naturally occurring HVR1 of human antibodies recognizing the mimotopes.

The mimotopes were used as immunoadsorbents to purify the specific antibodies from the bulk of anti-HVR1 present in infected patients' sera. Mimotopes R9, F78, M122, R6, B14, G31, H1 and D6 (Figure 7) were chosen from those which displayed the highest frequency of reactivity with the HCV sera. Mimotope N5, which was recognized by a significantly lower percentage of HCV sera (35%) than the average 'good' mimotopes (60–80%), was also used. Although some lymphocyte cell lines have been shown to support limited replication of HCV (Shimizu *et al*., 1992), these systems are not suitable for viral propagation or detailed study of the cross-reactivity of anti-HVR1 antibodies. Therefore, we determined the cross-reactivity of the immunopurified antibodies on a panel of synthetic peptides reproducing natural HVR1 variants which approximately cover the observed sequence variability. To this end, a multi-dimensional cluster analysis (Casari *et al*., 1995) was performed on the same set of 234 aligned natural HVR1 sequences used for the construction of the library. Of these, 43 sequences nearly homogeneously distributed over the HVR1 'sequence space' were chosen (see Materials and methods) and synthesized as multiple antigenic peptides (MAP; Pessi *et al*., 1990). A pool of eight sera from infected patients, that collectively reacted with the entire panel of 43 MAPs (data not shown), was used as a source of antibodies. The

A

mimotope	sequence
N5	TTTTTGGVQGHTTRGLVRLFSLGSKQN
R6	TTTTTGGQVGHQTSGLTGLFSPGAQQN
D6	QTTTTGGQVSHATHGLTGLFSLGPQQK
R9	QTTVVGGSQSHTVRGLTSLFSPGASQN
H1	QTHTTGGVVGHATSGLTSLFSPGPSQK
G31	TTHTVGGSVARQVHSLTGLFSPGPQQK
M122	QTTTTGGSASHAVSSLTGLFSPGSKQN
B14	QTTVTG-QASHTTSSLTGLFSPGASQK
F78	QTHTTGGQAGHQAHSLTGLFSPGAKQN

B

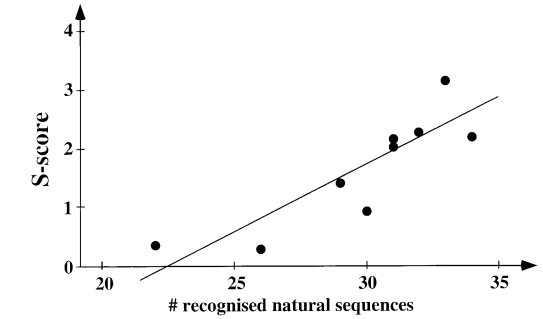

Fig. 7. Correlation between mimotope sequence and cross-reactivity. (**A**) Sequence of the mimotopes used in the analysis. (**B**) Correlation between the S-score of the clones and their cross-reactivity with a panel of 43 natural HVR1 sequences. The straight line represents the linear least square fit of the data. The correlation coefficient is 0.86.

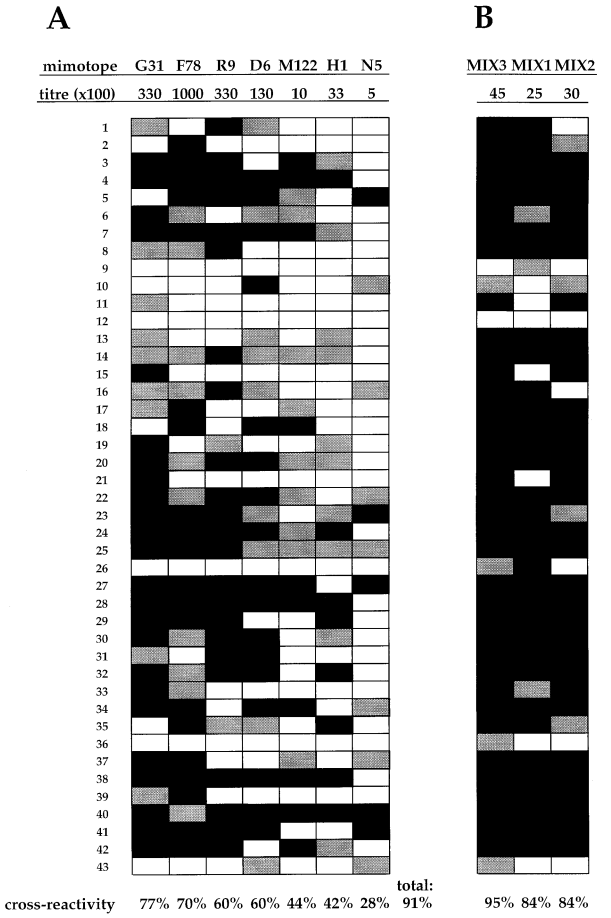

Fig. 8. The selected mimotopes are immunogenic mimics of a large number of naturally occurring HVR1. Reactivity of sera from mice immunized with single HVR1 mimotopes (**A**) and mixtures of mimotopes (**B**) in MAP form was assayed by ELISA on the panel of natural HVR1 sequences (left column). Immunizing mimotopes are shown in the first row. MIX1 includes mimotopes R9, F78, H1 and D6; MIX2 contains M122 and G31 peptides; MIX3 is composed of all six MAPs. Titres are shown in the second row. Sera were diluted 1:100. Average values from two independent experiments have been determined. Values were considered statistically significant when differing $>3\sigma_{max}$ ($P < 0.003$) from the background signal observed on two unrelated peptides. Grey boxes indicate signals differing from those observed on the unrelated MAPs between 0.15 and 0.5 OD (405 nm); black boxes indicate values differing more than 0.5 OD (405 nm). The level of cross-reactivity of each serum is indicated at the bottom of each column.

immunopurified antibodies displayed the same reactivity as the total serum to the mimotope used for the purification, whereas no reactivity to a recombinant HCV core antigen or to the antigens included in a commercially available kit **(**Materials and methods) was retained after purification, thus proving the efficiency and specificity of the purification (data not shown).

All immunopurified antibodies reacted with a significant number of natural HVR1 sequences, with mimotope R9 yielding antibodies that cross-reacted with 79% of natural HVR1 (Figure 6). As most immunopurified antibodies also displayed some non-overlapping reactivities to the natural sequences, an even higher level of overall cross-reactivity (88%) can be reached by adding up the individual contributions of antibodies purified from only three different mimotopes (R9, F78 and M122, Figure 6). From these data we concluded that a limited set of HVR1 mimotopes can antigenically mimic a large number of natural HCV HVR1 variants.

Interestingly, antibodies immunopurified by mimotopes with a higher S-score, and consequently a higher frequency of reactivity, were also more cross-reactive. Although the number of data points is not very high (only nine mimotopes were used, Figure 7A), the correlation between this sequence related score and the cross-reactivity of the corresponding antibodies is very good ($r = 0.86$; Figure 7B).

HVR1 mimotopes induce antibodies that recognize many natural HVR1 variants

The immunogenic potential of some of the best HVR1 mimotopes (R9, F78, M122, G31, H1 and D6) was investigated by injecting them into mice both as whole purified phage and, outside of the original context in which they were selected, as MAPs.

MAPs turned out to be much more potent immunogens, presumably due to the insufficient loading of HVR1 peptides on each phage as indicated by mass spectrometry analysis (<1% of the total pVIII content; data not shown). Some variability was observed in immunization level among the mimotopes, as shown by the difference in titer, with F78 inducing antibody titers >1:100 000 as measured by ELISA (Figure 8A). Anti-HVR1 mimotope sera were then tested for their ability to recognize heterologous HVR1 variants by ELISA on the previously described panel of 43 MAPs reproducing HCV sequences from natural isolates. Most of these MAPs were recognized by the immune sera (Figure 8A), while no reactivity was observed on unrelated control peptides (data not shown).

The ranking of the anti-mimotope mouse immune sera according to the level of cross-reactivity is different from that of human antibodies immunopurified over the same mimotopes. However, mimotope N5, which bound human antibodies showing low levels of cross-reactivity, was a much less efficient immunogen, leading to an anti-HVR1 response against a minority of the natural HVR1 sequences (Figure 8A).

The extent of cross-reactivity of the immune sera generally reflects the immunogenicity of the individual MAPs as, in most cases, a higher titer corresponds to a higher level of cross-reactivity (Figure 8A). Nevertheless, titer alone cannot always explain the differences in cross-reactivity and the pattern of reactivity, as clearly shown with the anti-G31 serum which has a lower titer than the anti-F78, but reacts with a larger number of natural HVR1 peptides. Similarly, the anti-D6 serum displays the same level of cross-reactivity as the anti-R9, despite a 3-fold lower titer (Figure 8A).

The reactivity pattern of each antiserum only partially overlaps that of the others, and in some cases is unique. As a consequence, when all reactivities are added, almost all natural HVR1 peptides are recognized (91%; Figure 8A). This observation prompted us to test whether a similar increase in cross-reactivity could be obtained by a single immunization with a cocktail of mimotopes. We thus immunized three groups of Balb/c mice with mixtures of mimotopes. Mixture 1 contained mimotopes R9, F78, H1 and D6; mixture 2 was composed of mimotopes M122 and G31; while mixture 3 comprised all six mimotopes. All three mixtures were immunogenic, and induced highly cross-reactive antisera (Figure 8B). Each of the three antisera displayed a cross-reactivity that was similar or even higher than the sum of reactivities of the antisera induced by each of the mimotopes in the mixture (84 versus 84% for MIX1, 84 versus 81% for MIX2 and 95 versus 91% for MIX3; Figure 8B). Although high, the titers of these sera were not better than those obtained with individual MAPs. We therefore concluded that the induction of highly cross-reacting responses is not simply a consequence of the level of immunization.

Discussion

Antibodies against a single E2 HVR1 can neutralize the virus, but only in an isolate-specific manner (Farci *et al*., 1996). This finding provides a viable explanation for the lack of viral clearance, despite the strong anti-HVR1 humoral response in chronically infected HCV patients. One could envisage the following scenario: following infection, anti-HVR1 antibodies are most efficiently induced by the major variants. However, this response could be insufficiently broad-reacting and allow those variants bearing HVR1 with low affinity for the induced antibodies (presumably the less abundant and most divergent ones in the initial inoculum) to escape neutralization, to infect and subsequently to emerge as a different quasispecies. This model, based on the relative fitness of the HVR1 to the immunological environment, is supported by three lines of evidence. The first is the lack or delayed appearance of antibodies able to bind certain variants which become predominant during chronic infection in both humans and primates (Kato *et al*., 1993; Kojima *et al*., 1994). The second is the direct relationship between the presence of anti-HVR1 antibodies and the mutation frequency of HVR1, as witnessed by the lack of variation in immunodeficient HCV-infected humans and chimpanzees (Kumar *et al*., 1994; Odeberg *et al*., 1997; Shimizu *et al*., 1997; Toyoda *et al*., 1997). Finally, in contrast to the dynamic distribution of mutants during infection *in vivo*, the composition of HCV quasispecies remains identical when propagated *in vitro* (Ito *et al*., 1996).

If this model is correct, the major task in developing an effective vaccine strategy to HCV would be to induce a broadly cross-reacting anti-HVR1 response. One step in this direction was provided by the observation that in some cases sera from viremic patients contain anti-HVR1 antibodies recognizing variants retrieved from different patients (Scarselli *et al*., 1995). Given the extraordinary heterogeneity of inter- and intra-individual HVR1 sequences, this finding implies that a significant proportion of the naturally occurring variants induce antibodies to cross-react with different HVR1. Our analysis of >200 unique HVR1 sequences clearly indicated a pattern of conserved residues in the hypervariable portion of E2, suggesting that some functional and/or structural constraints exist in this region, and thus explaining the observed cross-reactivity of some antibodies.

Our strategy to demonstrate this hypothesis was therefore to search for improved versions of the putative HVR1 element(s) responsible for the observed cross-reactivity. We achieved this through the molecular repertoire approach, namely the selection of HVR1 mimotopes from a large collection of peptides using sera from infected patients. We reasoned that the higher the frequency of reactivity of the selected mimotopes with HCV sera, the broader their antigenic and immunogenic mimicry of natural variants would be.

The design of the library was guided by the assumption that variability observed in the available HVR1 sequences reflects the possible mutation range accessible to the virus. This hypothesis seems to be confirmed by the observation that HVR1 sequences, deposited in sequence databases after our analysis was completed, gave essentially the same consensus (data not shown). The complexity of the library had to be kept within the practical limits of present cloning techniques, thus a subset of the most frequent residues at each position, accounting for ~80% of the total, was included in the design. Notwithstanding this, the resulting library had a complexity (10^8) comparable with that of the natural repertoire of antibodies present in those infected individuals used for the selections (Winter and Milstein, 1991).

The rationale of the selection procedure was to increase the probability of identifying the mimotopes that most frequently reacted with different HCV sera. In fact, re-selecting phage with a patient serum different from that used for the first enrichment allows only those peptides recognized by antibodies of 'common' specificity between the two sera to be identified (Folgori *et al*., 1994; Mecchia *et al*., 1996; Prezzi *et al*., 1996). This was indeed the case; clones randomly chosen from pools of phage after two rounds of selection showed a significantly higher frequency of recognition by HCV sera than those isolated after one. As a result of this selection procedure and the design of the library, mimotopes binding to antibodies not

related to HCV were also frequently selected, but were easily counter-screened using sera from a control population. A large number of HCV-specific mimotopes were identified, most of which reacted with antibodies present in the majority of the sera from HCV-infected viremic patients (up to 80% of the tested samples). This percentage is far greater than those previously observed with synthetic peptides reproducing HVR1 sequences of *sensu lato* HCV isolates (~50%; Scarselli *et al*., 1995). This high frequency of reactivity is an intrinsic property of the selected mimotopes, as it was also measured using sera that had never been used either for the selection or screening procedures.

As previously shown, synthetic peptides reproducing HVR1 sequences from natural isolates are rarely, if at all, recognized by antibodies present in the sera from HCV-seropositive non-viremic individuals (Scarselli *et al*., 1995). In contrast, the HVR1 mimotopes were recognized by sera from these patients, though less frequently than by viremic sera. These findings are in good agreement with the hypothesis that HVR1 mimotopes have a higher capability of interacting with different anti-HVR1 antibodies than natural HVR1 variants. This is understandable since the latter were selected *in vivo* for escaping cross-reactivity to antibodies against heterologous HVR1, rather than for being able to bind them.

Probing human antibodies immunopurified over different HVR1 mimotopes against a large panel of peptides reproducing natural HVR1 sequences revealed that the selected mimotopes antigenically mimic a great number of viral variants. We believe that this property is directly correlated to the frequency of reactivity with patients' sera and hence it is a result of the selection procedure. In fact, the same hierarchy is observed when ranking the mimotopes according to their frequency of reactivity with HCV sera or to the level of cross-reactivity of the anti-mimotope immunopurified antibodies with natural HVR1. The observation that both the frequency of reactivity and the cross-reactivity are correlated to the sequence patterns of the clones implies that these are intrinsic properties of the recognized epitope.

Weak and strong clones differ most substantially in the C-terminal part of the HVR1 region, while the insertions, deletions and frame shifts found in some of the selected clones (which nonetheless react with many sera) cluster in the N-terminus. This indicates that the predominantly recognized epitope(s) is located in the C-terminal part of the HVR1 region, in line with a number of studies where shorter fragments within the HVR1 were tested for recognition by sera from HCV-infected patients (Zibert *et al*., 1997). However, the presence of a pattern of six discriminating positions distributed over a 22 residue segment strongly suggests that at least some of the antibodies recognize a more extended region within the HVR1 segment, in agreement with peptide mapping data (R.Roccasecca, unpublished data) Taking into account the number of highly conserved segments within HVR1, it would seem that the HCV HVR1, rather than being a truly variable segment, might actually adopt only one or a restricted spectrum of closely related conformations. From a structural point of view, this is reminiscent of the hypervariable loops of immunoglobulins that, despite their sequence variability, can adopt only a limited set of conformations, called canonical structures. These are determined by the loop length and by the presence of a few key residues in the loop and/or in the framework (Chothia and Lesk, 1987; Tramontano *et al*., 1990). Although the details of the HVR1 structure conformation will most likely vary to some degree between serotypes, it would seem sufficiently conserved to cause the observed cross-reactivity. In contrast to what is superficially suggested by its given name, hypervariable region 1, this N-terminal segment of the HCV E2/NS1 protein contains somewhat conserved elements, and we believe these are recognized and memorized by the host immune system.

The main goal of the present work was to identify synthetic surrogates of the HVR1 able to induce antibodies that reacted with virtually all HCV HVR1 variants. We selected mimotopes that antigenically mimic many natural variants, and are broad immunogenic mimics, as they also induced antibodies which cross-react with the majority of peptides from our panel of natural HVR1 variants. Comparing the levels of cross-reactivity of the induced antisera, a different hierarchy of the mimotopes was observed with respect to the one established on the basis of the frequency of reactivity with patients' sera and the binding of cross-reacting antibodies. This discrepancy could be expected in view of the difference in genetic background between humans and mice, and as a consequence of the different way the immune systems of humans and mice were challenged; a natural infection by the whole virus in the former case and an experimental immunization with a synthetic peptide in the latter. Nevertheless, a clear difference was observed both in the efficiency of recognition and the number of bound natural HVR1 sequences between the antisera, induced by the group of 'high quality' clones and that obtained by injection of mimotope N5, a 'low quality' clone, according to both the criteria of recognition frequency with human sera and binding to cross-reacting antibodies. Similar results were obtained by immunization of rabbits with the same form of immunogen (MAPs), or when plasmids encoding for the mimotopes were used to immunize different species (mice, rats and rabbits) by intramuscular or intradermal delivery (data not shown). It thus also appears that the property of raising highly cross-reacting anti-HVR1 antibodies is an in-built feature of each particular mimotope and could be selected for by the procedure utilized. A net increase in cross-reactivity against the natural HVR1 was obtained by using mixtures of mimotopes as immunogens. Our results also indicated that the more complex the mixture, the broader the response would be. In some cases cross-reactivity of the antisera raised using cocktails of mimotopes was even higher than that obtained by adding up the reactivities of the sera induced by the individual mimotopes contained in the mixture. We interpret these data as an indication of immunogenic synergy between the different mimotopes in the cocktail, whereby the emergence of new variants during disease could lead to interference in the development of broadly reacting antibodies. A mechanism of 'original antigenic sin', previously proposed to explain escape from immune surveillance by influenza virus and HIV (Nara *et al*., 1991; Dimmock, 1993), could be responsible for the lack of protection by anti-HVR1 antibodies following natural infection by HCV. As discussed above, compelling evid-

ence has started to accumulate for a selection mechanism of HCV escape mutants by specific anti-HVR1 antibodies in infected patients. One way to deal with such a problem could be to induce broadly cross-reactive antibodies by using our mimotopes to prevent outgrowth of escape mutants, rather than require the immune system to deal with them after they arise. As a first step toward the development of an anti-HCV vaccine and in the light of the recently published work on the *ex vivo* neutralization of an infectious HCV inoculum by anti-HVR1 antibodies (Farci *et al*., 1996), we plan to assess the efficacy of anti-HVR1 mimotope antisera in blocking all variants of different quasispecies using the same model system. Similarly, the generation of chimeric E2 glycoproteins, where the original HVR1 is replaced by the selected mimotopes, would yield the ideal immunogens for vaccination experiments in primates.

More generally, we believe that the strategy presented here is a feasible approach to the problem of antigen variability where cross-neutralizing antibodies recognize overall shapes rather than primary sequence. In these cases, such as for the V3 loop of HIV gp120 (Nara *et al*., 1991), the true common conformation could be decoded by selecting specialized libraries with the natural repertoire of antibodies elicited by large numbers of variants of the infecting microorganism.

Materials and methods

Human sera

Human sera from HCV-infected patients and from healthy individuals were characterized for the presence of antibodies to HCV by a second-generation HCV ELISA test system (Ortho-HCV ELISA, Ortho Diagnostic Systems, Bersee, Belgium) and by a first generation dot-blot immunoassay (RIBA-HCV test, Chiron Co., Emeryville, CA). The presence of HCV RNA was detected by nested reverse transcription–PCR using conserved primers localized in the 5′ non-coding region of the viral genome, and total RNA was extracted from 100 µl of serum as previously described (Silini *et al*., 1995).

Construction of the HVR1 library

To back-translate the consensus profile described in the results section and in Figure 1B into the corresponding nucleotide sequence, the *Escherischia coli* codon usage table was employed, selecting the most frequent codons in highly expressed genes. To facilitate insertion of the library into the phagemid vector, two additional constant sequences containing the recognition sites for the restriction enzymes *Pac*I and *Not*I were added 5′ and 3′, respectively, to the 81 bp segment, giving a total of 116 bp. Absence of *Not*I and *Pac*I restriction sites in the back-translation of the consensus profile was verified by computer-assisted sequence analysis. A codon-based 'split-and-pool' method (Cormack *et al*., 1993) was applied for the chemical synthesis in order to keep both library composition and complexity at the desired level. The 116 bp oligonucleotides were amplified with primers complementary to the flanking constant sequences in a 9600 DNA Thermal Cycler (Perkin-Elmer Cetus, Foster City, CA). The PCR product was digested with *Pac*I and *Not*I enzymes and gel-purified. The recovered DNA fragment was cloned between the *Pac*I and *Not*I sites of the pel8PN phagemid vector (a derivative of pc89; Felici *et al*., 1991) downstream of the pelB secretion leader and upstream of the entire gene VIII coding sequence. Recombinant phagemids were electroporated into DH10B competent cells. Since DH10B cells cannot be infected by filamentous phage and do not allow for blue/white selection, transformed cells were collected and plasmid DNA was prepared. This DNA was used to transform XL1-blue competent cells by electroporation. Ampicillin resistant colonies were scraped from the plates and resuspended in LB/100 µg ampicillin/ml and 10% (v/v) glycerol. A portion of this bacterial suspension was inoculated into 6 l of LB medium containing 100 µg ampicillin/ml at 0.05 OD_{600} and grown with vigorous shaking until 0.25 OD_{600} was reached. The culture was then superinfected with M13K07 helper phage and grown for an additional 5 h to obtain the phage particles in the supernatant. The phage were precipitated twice with polyethylene glycol and purified by equilibrium centrifugation in CsCl as described (Felici *et al*., 1991).

DNA sequencing was performed as described (Bartoli *et al*., 1996) using an Applied Biosystem 373 DNA sequencer.

Library affinity selection

ELISA multiwell plates (Nunc Maxisorp, Roskilde, Denmark) were coated overnight at 4°C with 0.5 µg/ml of anti-human (Fc-specific) polyclonal Ab (Immunopure goat anti-human IgG Fc-specific; Pierce, Rockford, IL) in 50 mM $NaHCO_3$ pH 9.6. The plates were washed with PBS/0.1% Tween 20 (washing buffer) and incubated for 1 h at 37°C with 100 µl/well of blocking buffer (5% non-fat dry milk, PBS/0.05% Tween 20). Human serum (1 µl) diluted 1:100 in PBS/0.1% BSA was added to each well and incubated overnight at 4°C. After washing, 10^{12} particles of UV light killed M13K07 diluted in PBS/0.1% Tween 20. BSA (0.01%) was then added to each well and incubated for 4 h at 4°C. After this pre-incubation, 10^{12} particles/well of HVR1 library were added and incubated overnight at 4°C. Unbound phage were removed and several rounds of washing were performed. Bound phage were eluted with 200 µl of elution buffer (0.1 M HCl adjusted to pH 2.5 with glycine, 1 mg/ml BSA) and neutralized with 2 M Tris–HCl pH 9. Eluted phage were titrated by infection of XL1-blue bacteria and the percentage of clones containing a productive insert was determined by plating infected bacteria on X-gal/IPTG indicator plates (Felici *et al*., 1991). After amplification (see above) enriched phage were subjected to a second cycle of affinity selection following the same procedure.

Sequence analysis of the mimotopes and definition of the S-score

Out of a total of 200 selected clones, 180 showed no point mutation (with respect to the original library design) or deletions and were divided into three classes: 25 weak clones (reacting with less than three out of the 20 tested sera), 29 strong clones (reacting with at least 12 sera) and 126 intermediate clones.

For each amino acid at position i of a 27mer amino acid sequence, Fs(i, aa) and Fw(i, aa) represent the observed frequency of the same amino acid in position i of the sets of strong and weak clones, respectively.

We then define S-score(i) as the difference between the square roots of Fs(i,aa) and Fw(i,aa). The sum over the all 27mer sequence of S-score (**i**) is our sequence based S-score:

$$\text{S-score} = \Sigma_i \, [\alpha Fs(i,aa) - \alpha Fw(i,aa)]$$

where aa is the amino acid observed in position **i** of the sequence for which the S-score is calculated. The square root of frequencies was used to amplify differences. For clones where a point mutation or deletion had occurred, the corresponding position was omitted in the score calculation. The frequency values used are shown in Table II. The same analysis performed by including also the 20 clones with point mutations or deletions led to the same results.

Selection of a representative set of natural HVR1 sequences

The NS1 HVR1 sequence from the HCV BK strain (residues 384–411) was used to search both protein (SwissProt, PIR and Genpept, the latter representing assigned open reading frames from DDBJ/EMBL/GenBank) and nucleotide (DDBJ/EMBL/GenBank and EST) databases. Duplicated and incomplete sequences were removed from the list of matching sequences to obtain a unique set of 234 natural HVR1 sequences.

We used principle component analysis to select 40 sequences homogeneously distributed over the set. First, all pairwise distances between the 234 sequences were calculated using the first six eigenvalues calculated using the Sequencespace program (Casari *et al*., 1996). Sequences with the shortest distances to neighbouring sequences were eliminated in a stepwise procedure until only 40 sequences remained. Two dimensional projections along all possible pairs of Eigenvectors showed that the set of 40 sequences did not cluster and were homogeneously distributed. Accession numbers and sequences are shown in Table III. Three additional sequences were also synthesized as MAP: two sequences were derived from the pedigreed HCV inoculum H77 (Figure 2 of Farci *et al*., 1994):

41 (H77-1) ETHVTGGNAGRTTAGLVGLLTPGAKQN bp 1–81

42 (H79) ETHVTGGSAGHTAAGIASFFAPGPKQN bp 1–81

and one from the major isolate of a patient whose immunoreaction has been characterized (Scarselli *et al*., 1995):

43 GenBank: X79669 NTRVTGGVQSHTTRGFVGMFSLGPSQR bp 1–81.

Table II. Frequency values of the equation components used to calculate S-score

i	aa	Fs(i,aa)	Fw(i,aa)	i	aa	Fs(i,aa)	Fw(i,aa)	i	aa	Fs(i,aa)	Fw(i,aa)
1	Q	0.69	0.62	11	H	0.83	0.37	20	F	1.00	1.00
	T	0.31	0.38		R	0.17	0.63	21	S	1.00	0.17
2	T	1.00	1.00	12	Q	0.52	0.54		R	0.00	0.83
3	T	0.52	0.25		T	0.27	0.37	22	P	0.90	0.34
	H	0.48	0.13		A	0.21	0.21		L	0.07	0.29
	R	0.00	0.62	13	V	0.35	0.21		Q	0.03	0.29
4	T	0.69	0.50		A	0.34	0.25		S	0.00	0.08
	V	0.31	0.50		T	0.31	0.54	23	G	1.00	1.00
5	V	0.55	0.33	14	H	0.42	0.34	24	A	0.42	0.04
	T	0.45	0.67		S	0.41	0.33		S	0.38	0.17
6	G	1.00	1.00		R	0.17	0.33		P	0.21	0.79
7	G	1.00	1.00	15	S	0.52	0.33	25	K	0.42	0.34
8	Q	0.41	0.25		G	0.42	0.25		S	0.38	0.33
	S	0.31	0.58		R	0.07	0.42		Q	0.21	0.33
	V	0.28	0.17	16	L	1.00	1.00	26	Q	1.00	1.00
9	A	0.45	0.25	17	T	0.72	0.50	27	N	0.72	0.54
	Q	0.41	0.42		V	0.28	0.50		K	0.38	0.46
	V	0.14	0.33	18	G	0.48	0.37				
10	S	0.52	0.67		S	0.45	0.25				
	G	0.31	0.29		R	0.07	0.38				
	A	0.17	0.04	19	L	1.00	1.00				

Table III. Accession numbers and sequences

1	GenBank:D12967	QTRTVGGQMGHGVRGLTSLFSAGSARN	bp 46–126
2	PIR:PC1193	STHVTGALQGRAAYGITSFLSHGPSQK	aa 16–42
3	GenBank:D00574	HTRVTGGVQGHVTSTLTSLFRPGASQK	bp 1240–1320
4	GenBank:L19383	ETHTSGGSVARAAFGLTSIFSPGAKQN	bp 46–126
5	GenBank:M62381	ETHVTGGSAGRTTAGLVGLLTPGAKQN	bp 1426–1506
6	GenBank:U24616	ATYTTGGSAAKTAHRLASFFTVGPKQD	bp 22–102
7	PIR:C48776	DTHVVGGATERTAYSLTGLFTAGPKQN	aa 13–39
8	GenBank:U24607	GTTCQGGVYARGAGGIASLFSVGANQK	bp 22–102
9	PIR:D48766	RTLSFGGLPGHTTHGFASLSAPGAKQN	aa 13–39
10	GenBank:X60573	RTILMAGRQAEVTQSFPGLFSLAPSQK	bp 46–126
11	GenBank:D43650	NTHAMGGVVARSAYRITSFLSPGAAQN	bp 1–81
12	PIR:PQ0835	STRITGGSMARDVYRFTGFFARGPSQN	aa 6–32
13[a]	GenBank:S73387	GTHTIGGSQAQQANRFVSMFSRGPSQK	aa 190–216
14	GenBank:D10934	NTYVTGGAAARGASGITSLFSRGPSQK	bp 1491–1571
15	GenBank:D31972	NTYASGGAVGHQTASFVRLLAPGPQQN	bp 1409–1489
16	GenBank:U14231	ETHTTGGEAARTTLGIASLFTSGANQK	bp 103–183
17	GenBank:U24602	ETHTTGGSAARATFGIANFFTPGAKQN	bp 22–102
18	GenBank:L19380	ETYTSGGSAAHTTSGFVSFFSPGAKQN	bp 46–126
19	GenBank:M74888	GTTRVGGAAARTTSSFASLLTHGPSQN	bp 1147–1227
20	GenBank:L12354	NTHTVGAAASRSTAGLTSLFSIGRSQK	bp 1468–1548
21	GenBank:X79672	NTRVTGGVQSRTTGTFVGLFTPGPSQR	bp 1–81
22	PIR:A48776	NTHVSGGRVGHTTRSLTSFFTPGPQQK	aa 13–39
23	GenBank:D12952	STRVSGGQQGRAAHSLTSLFTLGASQN	bp 46–126
24	GenBank:D16566	STRITAQAEGRGASTLTSLFTSGASQK	bp 8–88
25	GenBank:M84754	STIVSGGTVARTTHSLASLFTQGASQK	bp 1491–1571
26	GenBank:D14853	ETRVTGGAAGHTAFGFASFLAPGAKQK	bp 1491–1571
27	GenBank:S24080	NTYVTGGSAGRAVAGFAGLLQPGAKQN	bp 46–126
28	GenBank:S35631	ETHSVGGSAAHTTSRFTSLFSPGPQQN	bp 580–660
29	GenBank:S62395	ETHVTGGSAASTTSTLTKLFMPGASQN	bp 43–123
30	GenBank:S70291	QTRTVGGANARNTYGLTTLFTTGPKQN	bp 1–81
31	GenBank:D88472	GTTTVGSAVSSTTYRFAGMFSQGAQQN	bp 1485–1565
32	GenBank:D10687	NTHTVGGTEGFATQRLTSLFALGPSQK	bp 1180–1260
33	GenBank:D43651	NTHVTGGVVARNAYRITTFLNPGPAQN	bp 39–119
34	GenBank:D14305	HTYTTGGTASRHTQAFAGLFDIGPQQK	bp 1427–1507
35	GenBank:X60590	KTHVTGMVAGKNAHTLSSIFTSGPSQN	bp 46–126
36	GenBank:D30613	GTHVTGGKVAYTTQGFTSFFSRGPSQK	bp 1491–1571
37	GenBank:X53131	ETYTSGGNAGHTMTGIVRFFAPGPKQN	bp 802–882
38	GenBank:U24619	STYSMGGAAAHNARGLTSLFSSGASQR	bp 22–102
39	GenBank:M62382	ETHVTGGSAGRSVLGIASFLTRGPKQN	bp 1426–1506
40	GenBank:D88474	ETYIIGAATGRTTAGLTSLFSSGSQQN	bp 1488–1568

[a]Sequence 13 corresponds to the translated amino acid sequence (aa190–216) reported in the CDS feature of GenBank entry S73387.

Phage preparation and ELISA

Phage supernatants were prepared from XL-1 blue infected cells as previously described (Folgori *et al*., 1994). ELISA were performed according to Dente *et al*. (1994) using 25 µl of phage supernatant/well. Sera were diluted 1:100 if not otherwise specified and revealed by addition of species-specific anti-IgG (Fc-specific) alkaline phosphatase-conjugated secondary antibodies (Sigma A-9544; dilution 1:5000 in ELISA blocking buffer). Results were recorded as differences between OD_{405} and OD_{620} by an automated ELISA reader (Labsystems Multiskan Bichromatic, Helsinki, Finland).

ELISA with phage pools were performed in the same way by using equivalent amounts (10^{10} ampicillin transducing units) of amplified phage after CsCl purification (see above).

HVR1 MAPs (100 µl) were used to coat ELISA plates (Nunc Maxisorp, Roskilde, Denmark) at a final concentration of 10 µg/ml in coating buffer (50 mM $NaHCO_3$ pH 9.6). After blocking non-specific binding sites, 100 µl/well of sera or affinity-purified antibodies were added. Mouse and rabbit sera were tested at final 1:100 dilution in blocking buffer; affinity purified antibodies were tested at final concentration of 150 ng/ml. Plates were incubated overnight at 4°C. After washing, 100 µl/well of alkaline phosphatase conjugated secondary antibodies (goat anti-mouse IgG Sigma A-7434 diluted 1:2000; goat anti rabbit IgG Sigma A-8025 diluted 1:5000; goat anti human IgG Sigma A-9544 diluted 1:5000) were added and incubated for 1 h at room temperature. Plates were washed and alkaline phosphatase revealed as described above.

Affinity purification of antibodies from human sera

MAPs reproducing the sequence of different mimotopes were used since they showed the same binding profile with HCV sera in ELISA as the phage, but proved to be more efficient in the affinity selection of the antibodies (data not shown). Activated CH Sepharose 4B column (Pharmacia Biotech 17-0490-01) was coupled with the MAP of interest at the ratio of 1 g of dried sepharose/1mg of MAP in coupling buffer (0.1 M $NaHCO_3$ pH 8/0.5M NaCl). Coupling was followed by blocking free amino-groups with 0.1 M Tris–HCl pH 8. Sample was loaded as a pool of eight HCV sera diluted 1:5 in coupling buffer. After adsorption at room temperature and extensive washing with PBS, bound antibodies were eluted with 0.1 M glycine–HCl pH 2.7 supplemented with BSA at final concentration of 10 µg/ml and immediately neutralized by 2 M Tris–HCl pH 9.4. The concentration of eluted antibodies was determined by ELISA using human IgG (Sigma I-2511) as a standard. Affinity-purified antibodies were checked for their reactivity in ELISA with the mimotope used for purification (both in MAP and phage forms) and, as control, with HCV-unrelated MAPs. The specificity of the purification was further confirmed by testing the eluted antibodies by ELISA on recombinant bacterially expressed HCV core protein (Prezzi *et al*., 1996) and by the second-generation HCV ELISA test (Ortho Diagnostic Systems, Bersee, Belgium). The total amounts of immunoglobulins recovered in each affinity purification from a standard amount of 1 ml of serum pool were comparable, ranging from 0.8 to 1.5 µg. For ELISA on MAPs, the concentration was adjusted in each case to 150 ng/ml.

Animal immunization

Immunizing phage were prepared from XL1-blue infected cells and CsCl purified as previously described (Felici *et al*., 1991). Three to five week-old female Balb/c mice (Charles River, Como, Italy) were immunized by intraperitoneal injection of 100 µl of antigen solution at day 0, 21 and 42 and bled at day 52 (third bleed) and day 148 (fourth bleed). Phage were injected as 0.9% NaCl suspensions at a concentration of ~0.3 mg/ml (2.5×10^{13} phage particles/ml) without added adjuvant.

For peptide immunizations, MAPs were dissolved in PBS at a final total concentration of 400 µg/ml and injected as a 1:2 dilution in either Complete Freund's Adjuvant (first injection) or Incomplete Freund's Adjuvant (booster injections). Four to seven week-old female Balb/c mice (Charles River, Como, Italy) were immunized by i.p. injection of 100 µl of antigen solution at weeks 0, 3 and 6 and bled at days 0 (pre-bleed) and 10 days after each additional injection. When more than one peptide was used for immunization, equal amounts of each mimotope were mixed, and 100 µl of a 400 µg/ml solution was used.

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