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Immunogenicity of variable regions of hepatitis C virus proteins: selection and modification of peptide epitopes to assess hepatitis C virus genotypes by ELISA

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The immunogenicity of variable regions of hepatitis C virus (HCV) proteins was studied by ELISA by using 543 synthetic peptides from 120 variable regions and 90 sera from HCV-infected patients. Some regions from certain genotypes were less immunogenic, or even non-immunogenic, compared with their equivalents in other genotypes. However, the mean recognition of all peptides from genotypes 1a, 1b and 3 by sera infected with genotypes 1a, 1b and 3, respectively, showed no significant differences, suggesting a similar overall immunogenicity of variable regions from these genotypes. Proteins NS4a, NS4b and NS5a were found to be the most immunogenic. Recognition of individual peptides by the sera of infected patients showed that the humoral response against HCV is patient-dependent. The work shows that 15-mer peptides may encompass several B-cell epitopes. These epitopes may lie in slightly different positions in different genotypes. Thirty-one percent of the 543 peptides were recognized by some of the 35 healthy donors. This may be a reflection of the large number of antigens to which they had been exposed, but it may also reflect a strategy of HCV to respond to immune pressure. After selection and modification, a set of 40 peptides was used to assess genotypes 1a, 1b, 1, 2 and 3 in the sera of HCV-infected patients, with sensitivities of 34·1, 48·5, 68·8, 58·3 and 48·9% and specificities of 100, 99·1, 97·1, 99·5 and 99%, respectively. The overall sensitivity and specificity for the assessment of genotypes 1, 2 and 3 were 64 and 98%, respectively.

Introduction

Hepatitis C virus (HCV) is a highly prevalent virus worldwide. Its prevalence in developed countries is around 1% of the general population (Rall & Dienstag, 1995) and around 0·1–0·7% in blood donors (Shakil *et al.*, 1995). This prevalence may be as high as 6% in some regions of Africa and the Middle East (Darwish *et al.*, 1993). Since HCV is mainly transmitted via blood, haemo-derivatives and haemodialysis, the main populations at risk are drug addicts, the recipients of blood transfusions and of haemodialysis (Alter, 1989), organ transplants (Pereira *et al.*, 1991) and tattoos (Ko *et al.*, 1992) and health-care personnel (Polish *et al.*, 1993). Approximately 80% of patients suffering from acute hepatitis C develop chronic hepatitis (Lau *et al.*, 1993) and 20–35% develop liver cirrhosis (Alter, 1989). A significant proportion of chronic patients develop hepatocellular carcinoma (Tsukuma *et al.*, 1993).

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HCV was first identified and cloned by Choo *et al.* (1989). This allowed the engineering of ELISA assays based on recombinant proteins (Kuo *et al.*, 1989) and synthetic peptides (Berasain *et al.*, 1993) and more recently on a combination of recombinant proteins and synthetic peptides (Vrielink *et al.*, 1995). Soon after the first complete genome sequence of HCV was reported (Choo *et al.*, 1991), two other sequences from Japanese isolates were published (Kato *et al.*, 1990; Takamizawa *et al.*, 1991). Several types and subtypes of HCV have been found in different geographical areas (Bukh *et al.*, 1993). Today, the most widely accepted classification of HCV genotypes is that put forward by Simmonds *et al.* (1994), which allows the definition of six virus types and a great number of subtypes. The nucleotide sequence identity between types is around 66–69% and that between subtypes of the same type 77–80%, whereas an identity of 91–98% is found between sequences of the same virus subtype (Simmonds, 1995). HCV genotypes 1a, 1b, 2a, 2b and 3 are distributed worldwide, whereas types 4, 5 and 6 are found mainly in North and Central Africa, South Africa and south-west Asia, respectively (Bukh *et al.*, 1993).

Table 1. Synthetic peptides used in this study

The table shows the location of each variable region selected for this study [name of protein and location within the HCV sequence as given by Choo *et al.* (1991), accession number M62321]. The source of the sequences for each genotype is also given; the GenBank accession numbers or references are: 1, M62321; 2, M67463; 3, X53134; 4, D10749; 5, L23439; 6, M96362; 7, D90208; 8, M74814, 9, D13558; 10, D10687; 11, L02836; 12, D11168; 13, D10750; 14, D00944; 15, L16650; 16, D10075; 17, M95105; 18, M95103; 19, L16655; 20, D10077; 21, L16672; 22, D01221; 23, Tsukiyama-Kohara *et al.* (1991); 24, Simmonds *et al.* (1993) (T0059); 25, D10650; 26, D17763; 27, D14063; 28, L16659; 29, D10115; 30, Simmonds *et al.* (1993) (T0040); 31, D10078; 32, D10080. ND, Not done.

		Source of sequence							Source of sequence				
No.	HCV region	1a	1b	2a	2b	3	No.	HCV region	1a	1b	2a	2b	3
1	Core 67–78	1	6	14	ND	26	61	NS4b 1930–1943	1	ND	14	ND	ND
2	Core 156–165	1	ND	ND	ND	27	62	NS4b 1946–1959	1	7	14	22	26
3	E1 192–204	1	7	15	19	26	63	NS4b 1958–1971	1	7	14	22	26
4	E1 210–223	1	7	14	19	26	64	NS5a 1982–1995	1	6	14	22	26
5	E1 214–224	1	7	ND	ND	ND	65	NS5a 1996–2009	1	6	14	22	26
6	E1 216–229	1	6	14	19	26	66	NS5a 2022–2035	1	7	14	22	26
7	E1 246–260	2	8	14	20	26	67	NS5a 2034–2047	1	7	14	22	26
8	E1 262–275	1	6	14	20	26	68	NS5a 2045–2058	1	7	14	22	26
9	E1 283–296	1	7	14	21	26	69	NS5a 2062–2075	1	7	14	22	26
10	E1 307–320	1	7	14	20	26	70	NS5a 2076–2089	1	7	14	22	26
11	E1 310–324	1	7	ND	ND	ND	71	NS5a 2092–2105	1	7	14	22	26
12	E1 314–326	1	7	ND	ND	ND	72	NS5a 2121–2134	1	7	14	22	ND
13	E1 330–344	3	7	16	20	26	73	NS5a 2136–2149	1	13	14	22	26
14	E1 345–360	1	7	16	20	28	74	NS5a 2142–2155	1	12	14	22	26
15	E1 361–374	1	7	14	20	26	75	NS5a 2216–2229	1	7	14	22	26
16	E2 403–416	4	9	ND	ND	ND	76	NS5a 2240–2253	1	ND	14	22	26
17	E2 408–421	2	7	ND	ND	ND	77	NS5a 2256–2269	1	12	14	22	26
18	E2 456–469	1	10	14	22	26	78	NS5a 2262–2275	1	ND	14	22	26
19	E2 526–539	3	7	14	20	26	79	NS5a 2274–2287	1	12	14	22	26
20	E2 570–583	3	11	14	22	26	80	NS5a 2296–2309	1	7	14	22	26
21	E2 667–681	2	7	14	20	26	81	NS5a 2303–2319	1	13	14	22	26
22	E2 704–717	1	7	14	20	26	82	NS5a 2310–2323	1	13	14	22	26
23	NS2a 758–771	1	9	14	22	26	83	NS5a 2324–2337	1	7	14	22	26
24	NS2a 782–795	1	7	14	22	26	84	NS5a 2336–2349	1	7	17	22	26
25	NS2b 810–823	1	7	14	ND	26	85	NS5a 2343–2356	1	7	18	22	26
26	NS2b 822–835	1	7	14	22	26	86	NS5a 2350–2363	1	7	14	22	26
27	NS2b 836–849	1	7	14	22	26	87	NS5a 2357–2370	1	12	14	22	26
28	NS2b 852–865	1	7	14	22	26	88	NS5a 2364–2377	1	9	14	22	26
29	NS2b 872–885	1	7	14	22	26	89	NS5a 2371–2384	1	7	14	22	26
30	NS2b 896–909	1	7	14	22	26	90	NS5a 2378–2391	1	7	14	22	26
31	NS2b 918–931	1	7	14	22	26	91	NS5a 2400 (Ins1*)	ND	ND	14	22	ND
32	NS2b 934–947	1	7	14	22	26	92	NS5a 2400 (Ins2*)	ND	ND	14	22	ND
33	NS2b 978–991	1	11	14	22	26	93	NS5a 2406–2419	1	13	14	22	26
34	NS2b 1012–1025	1	6	14	22	26	94	NS5b 2432–2445	1	12	14	22	26
35	NS3 1052–1065	1	6	14	22	26	95	NS5b 2451–2464	1	12	14	22	ND
36	NS3 1066–1079	1	7	14	22	26	96	NS5b 2478–2491	1	7	14	ND	26
37	NS3 1086–1099	1	7	14	22	26	97	NS5b 2492–2505	1	12	14	22	26
38	NS3 1102–1115	1	7	14	22	26	98	NS5b 2518–2531	1	12	14	22	26
39	NS3 1116–1129	1	6	14	22	26	99	NS5b 2529–2542	2	12	14	22	26
40	NS3 1142–1155	1	ND	14	22	26	100	NS5b 2565–2578	1	ND	14	22	26
41	NS3 1198–1211	1	7	14	22	26	101	NS5b 2598–2611	2	12	14	22	ND
42	NS3 1316–1329	1	7	14	22	26	102	NS5b 2618–2631	1	12	14	22	26
43	NS3 1362–1375	1	7	14	22	26	103	NS5b 2655–2668	1	12	14	22	26
44	NS3 1402–1415	1	7	14	23	26	104	NS5b 2662–2675	1	12	14	20	26
45	NS3 1452–1465	1	ND	14	23	26	105	NS5b 2680–2693	1	12	14	22	26
46	NS3 1494–1507	1	12	14	23	26	106	NS5b 2702–2715	1	ND	14	22	26
47	NS3 1577–1590	1	6	14	22	29	107	NS5b 2720–2733	1	7	16	25	32

Table 1 (*cont.*)

No.	HCV region	Source of sequence 1a	1b	2a	2b	3	No.	HCV region	Source of sequence 1a	1b	2a	2b	3
48	NS3 1646–1659	1	ND	14	23	26	108	NS5b 2743–2756	1	12	14	22	26
49	NS4a 1690–1699	5	7	14	24	26	109	NS5b 2792–2805	1	7	14	22	26
50	NS4a 1695–1708	1	12	14	22	26	110	NS5b 2832–2845	1	7	14	22	26
51	NS4b 1714–1727	ND	7	14	22	26	111	NS5b 2850–2863	1	7	14	22	26
52	NS4b 1718–1728	ND	ND	14	ND	ND	112	NS5b 2862–2865	1	7	14	22	26
53	NS4b 1728–1741	1	7	14	22	30	113	NS5b 2895–2908	1	7	14	22	ND
54	NS4b 1734–1743	ND	ND	ND	ND	26	114	NS5b 2914–2927	1	7	16	22	26
55	NS4b 1744–1757	1	7	14	22	30	115	NS5b 2930–2943	1	7	14	22	26
56	NS4b 1752–1758	ND	ND	ND	ND	26	116	NS5b 2958–2971	1	7	14	22	26
57	NS4b 1794–1807	1	7	14	ND	26	117	NS5b 2968–2981	1	13	14	22	26
58	NS4b 1816–1829	1	7	14	ND	26	118	NS5b 2983–2996	2	7	14	22	26
59	NS4b 1829–1842	1	7	14	ND	26	119	NS5b 2990–3003	2	7	14	22	26
60	NS4b 1868–1881	1	7	14	22	26	120	NS5b 2998–3011	1	7	14	22	26

* Sequences 14 and 22 (as listed in the legend) include a 20 aa insertion at position 2400. Region 91 (Ins1) corresponds to amino acids 1 to 14 and region 92 (Ins2) corresponds to amino acids 10 to 20 of this insertion.

The reasons behind this distribution are not well understood, although it has been postulated recently that the different HCV genotypes evolved from a common ancestor 500–2000 years ago (Smith *et al.*, 1997).

Different HCV genotypes may show differences in immunogenicity. Thus, antigens from HCV type 1 (used in commercial ELISA assays), from relatively conserved core and NS3 protein regions, may recognize serum antibodies from other genotypes with different sensitivities. For instance, it has been reported that antigens from these regions of virus genotype 1 are recognized five times more strongly by sera from patients infected with virus type 1 than by sera from those infected with virus genotypes 2 and 3 (Dhaliwal *et al.*, 1996). Virus genotypes may also influence the response to interferon-α (IFN-α) therapy. Thus, patients infected with HCV genotypes 1b and 4 seem to respond less efficiently than those infected with genotypes 2a, 2b and 3a (Yoshioka *et al.*, 1992; Hino *et al.*, 1994; Tsubota *et al.*, 1994; Dusheiko *et al.*, 1994; Yamada *et al.*, 1994; Hopf *et al.*, 1996). Although it has been reported that patients infected with virus genotypes 1a and 1b respond poorly to treatment compared with those infected with genotypes 2 and 3 (Davis & Lau, 1997), it has also been suggested that patients infected with genotype 1a may respond better than those infected with genotype 1b (Lohr *et al.*, 1996; Zein *et al.*, 1996; Sakugawa *et al.*, 1997; Bell *et al.*, 1997). However, it is not clear if this enhanced response is statistically significant. Virus genotype 1b is associated with hepatic damage and cirrhosis (Pozzato *et al.*, 1994) and is highly prevalent in patients who require hepatic transplantation (Zein *et al.*, 1995*a*) and in most cases of hepatocellular carcinoma (Zein *et al.*, 1995*b*; Takada *et al.*, 1996). The correlation of certain virus genotypes with immunogenicity, response to IFN-α therapy, cirrhosis and hepatocellular carcinoma has prompted several research groups to develop methods of genotyping the virus. There are at present two main approaches to assess the genotype of HCV. The first approach is by reverse transcription of viral RNA from infected sera and amplification of a region of this RNA either by nested PCR with genotype-specific primers (Okamoto *et al.*, 1992) or by using universal primers followed by identification of the amplified gene either by the pattern of fragments obtained after digestion with restriction enzymes (Davidson *et al.*, 1995; Mellor *et al.*, 1995; Gournay *et al.*, 1995) or by hybridization with type-specific probes (Viazov *et al.*, 1994). The second approach uses genotype-specific synthetic peptides to detect antibodies in the sera of infected patients (Simmonds *et al.*, 1993; Machida *et al.*, 1992; Tsukiyama-Kohara *et al.*, 1993; Tanaka *et al.*, 1994). The sensitivity and specificity of the first approach is higher but it is only available to some laboratories, whereas the second approach is simpler and can be used in most laboratories. Although the use of the genotype-specific synthetic peptides reported by Simmonds *et al.* (1993) can identify most virus genotypes, it is unable to distinguish between subtypes 1a and 1b, which are the most abundant in the Western world. We speculated that the synthesis of a large number of synthetic peptides from the most variable regions of proteins from HCV types and subtypes might give a useful insight into the relative immunogenicity of HCV proteins from different genotypes. It might also, at the same time, identify new type- and subtype-specific peptides for the identification

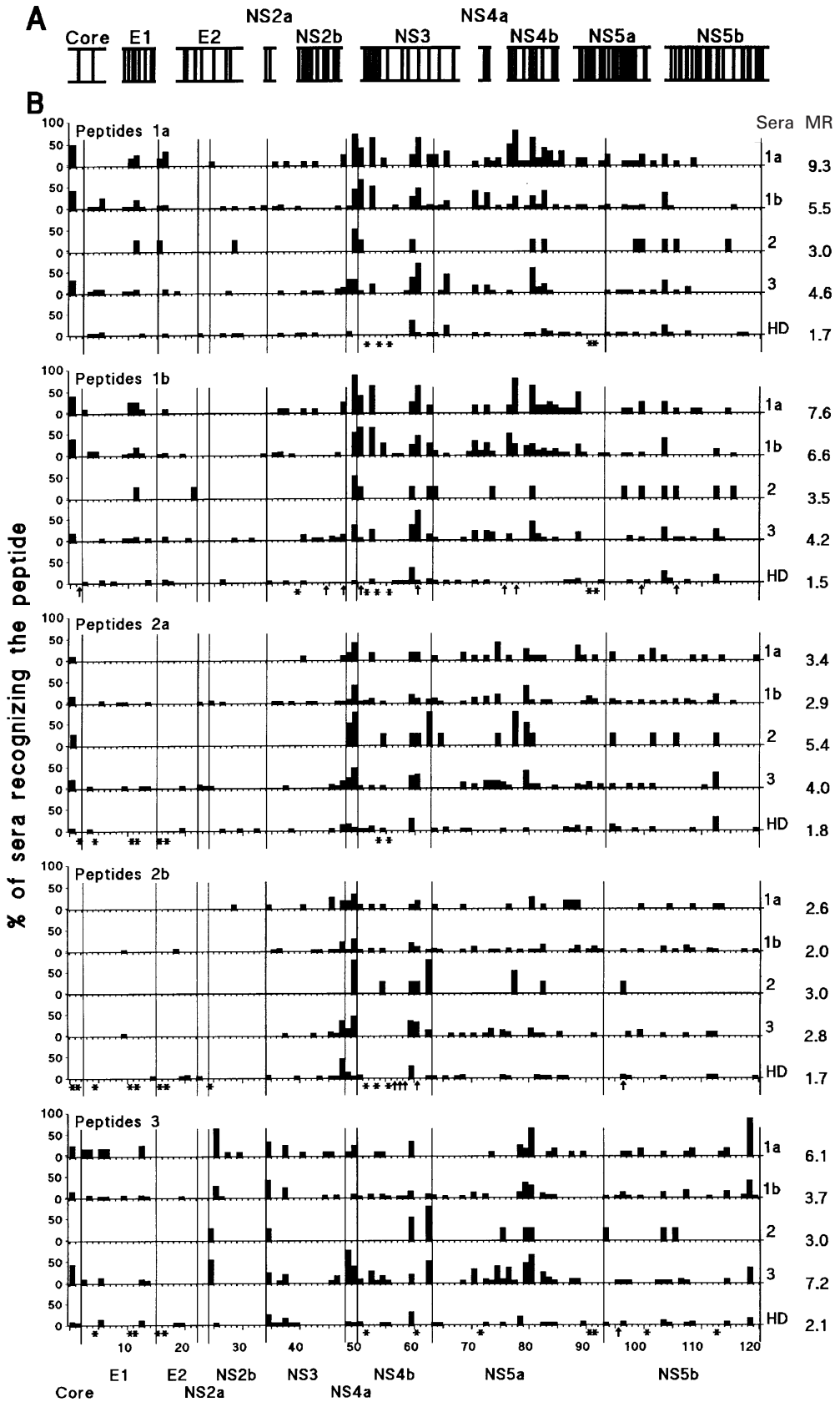

Fig. 1. For legend see facing page.

of genotypes 1, 2 and 3 and for differentiation between subtypes 1a and 1b. Moreover, a study of the recognition of this large number of peptides by the sera of HCV-infected patients with different responses to IFN-α therapy might be useful to predict response or non-response to this cytokine. Our findings in these areas are discussed.

Methods

■ **Peptides and peptide synthesis.** We synthesized 543 peptides from 120 regions of HCV proteins. These peptides were chosen after comparing 15 complete and 172 incomplete published HCV sequences corresponding to different genotypes. The exact locations of these 120 regions within the different proteins from genotypes 1a, 1b, 2a, 2b and 3 are given in Table 1. The peptides were selected from regions with important sequence diversity among genotypes, but with low or no diversity within the same type or subtype. Peptides were synthesized by the solid-phase method of Merrifield (1963) using the Nα-9-fluorenylmethoxycarbonyl (Fmoc) alternative (Atherton *et al*., 1981) and a multiple solid-phase peptide synthesizer (Borrás-Cuesta *et al*., 1991). In most cases, the peptides contained 15 amino acids and were all synthesized with an Ala at the C terminus for ease of synthesis. The peptides were used without further purification. Linear peptide homopolymers were prepared from side-chain-protected peptide monomers as described previously (Borrás-Cuesta *et al*., 1988). In brief, the peptide (5 μmol) was dissolved in 200 μl dimethylformamide (DMF) and the apparent pH was adjusted to pH 8–9 with N-ethyl morpholine (the pH was controlled by adding a drop of the solution on a wet piece of indicator paper). This solution was stirred during the addition of 5 μmol dicyclohexylcarbodiimide and 5 μmol 1-hydroxybenzotriazole dissolved in 100 μl DMF. After 48 h at room temperature, the reaction mixture was purified by gel filtration on a column of Sephadex LH-20 (Pharmacia) equilibrated in DMF. The polymers, recovered from the first peak, were dried under high vacuum and the amino acid side-chains were deprotected by treatment with 95% trifluoroacetic acid and scavengers. Peptides were precipitated with diethyl ether, dissolved in water and freeze-dried. The polymers thus obtained were used without further purification.

■ **Patients.** Four panels of sera from non-treated, HCV-infected patients and a panel of sera from healthy blood donors were used. The first panel consisted of 90 sera from our serum library (13, 46, four and 27 sera of patients infected with HCV genotypes 1a, 1b, 2 and 3, respectively). The second panel consisted of 101 sera from the Instituto Nacional de la Nutrición Salvador Zubirán, Mexico (13, 69, 16 and three sera of patients infected with HCV genotypes 1a, 1b, 2 and 3, respectively). The third panel contained another 54 sera from our serum library (14, 17, four, 15 and three sera of patients infected with HCV genotypes 1a, 1b, 2, 3 and 4, respectively). The fourth panel contained 14 sera from patients coinfected with different virus genotypes (four, five, three, one and one sera coinfected with HCV genotypes 1a + 1b, 1b + 4, 1b + 2, 1a + 3 and 2 + 3, respectively). All sera were positive against a third-generation ELISA (Ortho Diagnostics) and HCV RNA-positive by PCR. The fifth panel consisted of 35 sera from healthy blood donors with no clinical symptoms of hepatitis and without anti-HCV antibodies in their sera.

In order to correlate antibody peptide recognition with the outcome of IFN-α therapy, we tested the peptides by ELISA against the sera of 67 patients from the first panel, who were later submitted to IFN-α therapy as described by Gavier *et al*. (1997). The outcome of this therapy was: 34 sustained responders, six transient responders and 27 non-responders. We considered a patient as a sustained responder when, after treatment, their alanine aminotransferase level remained within the normal range (1–22 UI/l in women and 1–29 UI/l in men) and serum HCV RNA was persistently negative during the whole follow-up period (minimum follow up, 18 months). Transient responders were those who responded to IFN-α therapy but relapsed after IFN-α withdrawal. All other patients were considered as non-responders (Gavier *et al*., 1997).

■ **Detection of anti-peptide antibodies by ELISA.** Anti-HCV antibodies in the sera were detected by ELISA with synthetic peptides. Microtitre wells were coated by overnight incubation at 4 °C with 50 μl peptide solution (40 μg/ml peptide in 0·1 M sodium carbonate buffer, pH 10·5). Wells were then washed three times with 200 μl per well of a solution of PBST (2 mM KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, 0·1% Tween 20, pH 7·6). To block non-specific antibody binding, the wells were incubated with 300 μl per well PBSTM (PBST containing 1% powdered milk) for 1 h at room temperature. The wells were washed three times with 200 μl per well PBST and then 50 μl serum, diluted 1:20 in PBSTM, was added and incubated at 37 °C for 1 h. Wells were washed three times with 200 μl per well PBST and then incubated at 37 °C for 1 h with 50 μl of a solution of biotinylated goat anti-human IgG whole antibody (Amersham) diluted 1:1000 in PBSTM. After washing three times with 200 μl per well PBST, wells were incubated at 37 °C for 1 h with 50 μl of a solution of horseradish peroxidase–streptavidin (Amersham) diluted 1:500 in PBSTM. After washing three times with PBST, the colour reaction was started by adding 100 μl of a solution prepared by mixing 10 ml 0·6% acetic acid (pH 4·7), 7·5 μl 33% (w/v) hydrogen peroxide and 100 μl of a 40 mM aqueous solution of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid). After 1 h the absorbance was read at 405 nm in a Titretek Multiskan MKII. A serum was considered positive by ELISA against a peptide when the absorbance corresponding to this serum was at least twice the mean value of the 30 peptides giving the lowest absorbance within the same ELISA plate. The cut-off value thus obtained was similar for HCV-infected sera and for healthy sera.

■ **PCR amplification of serum HCV RNA and HCV genotyping.** HCV RNA was extracted from 100 μl serum by using the Ultraspec-RNA (Biotecx) extraction solution. Reverse transcription and nested PCR were carried out basically as described previously by Garson *et al*. (1990), but

Fig. 1. Peptides from variable regions of HCV proteins from genotypes 1a, 1b, 2a, 2b and 3 and their recognition by the first panel of 90 HCV-infected sera. (A) Diagram of the location of 120 regions (vertical lines) within the HCV proteins from which peptides were synthesized. (B) Peptides from a given genotype were tested against sera from the same genotype as well as against sera from the remaining genotypes and a panel of 35 sera from healthy donors (HD). Abscissa: position of the 120 variable regions (the location of peptides within the different HCV proteins is given in Table 1). Ordinate: percentage of sera recognizing the peptide. (*) Peptide was not synthesized; (↑) the sequence of peptides from genotypes 1a and 1b or 2a and 2b are identical. (MR) Mean recognition of peptides (see text). The genotype of the sera is indicated at the right-hand side of the figure.

Table 2. Examples of the effect of sequence displacement, shortening of peptides and substitution by Ala on peptide recognition by sera from patients infected with different HCV genotypes

Alanine residues included in peptides that are not contained in the wild-type sequence are indicated by A. Modified peptides are indicated by the suffix (mp). –, Not detected.

Amino acid sequence	Region of polyprotein	Genotype of peptide	Recognition by sera (%) 1a	1b	2	3	Controls
(i) Sequence displacement							
RAALIEEGQRIAEMA	NS4b 1714–1727	2a	–	2	–	4	3
ASRAALIEEGQRIA	NS4b 1712–1724 (mp)	2a	–	–	75	–	–
HLPYIEQGMQLAEQA	NS4b 1714–1727	1a,1b	38	65	25	4	–
YIEQGMMLAEQFKQA	NS4b 1717–1730 (mp)	1a	–	6	–	4	–
CSQHLPYIEQGMMLA	NS4b 1711–1725 (mp)	1a	92	85	25	37	14
(ii) Shortening of peptides							
EEDEREISVPAEILA	NS5a 2262–2275	1a,1b	61	26	–	–	3
AAEISVPAEILAAA	NS5a 2267–2275 (mp)	1a	23	–	–	–	–
ACKPLLREDVTFQVA	NS5a 2136–2149	1b	15	26	–	18	3
AAAADVTFQVAAAA	NS5a 2144–2149 (mp)	1b	61	35	75	37	26
RRLHQWISSECTTPA	NS4b 1958–1971	1a	23	6	–	–	3
AAQWISSECTTPAA	NS4b 1962–1971 (mp)	1a	–	–	–	–	–
KRLHQWINEDCSTPA	NS4b 1958–1971	1b	15	26	25	11	–
AAKRLHQWINEDAA	NS4b 1958–1967 (mp)	1b	–	6	–	11	–
AAQWINEDCSTPAA	NS4b 1962–1971 (mp)	1b	–	–	–	–	–
RRLHNWITEDCPIPA	NS4b 1958–1971	2a	–	6	75	–	–
AANWITEDCPIPAA	NS4b 1962–1971 (mp)	2a	–	2	50	–	–
RRLHAWITEDCPVPA	NS4b 1958–1971	2b	–	4	75	11	3
AAAWITEDCPVPAA	NS4b 1962–1971 (mp)	2b	–	–	50	–	–
RRLHQWINEDYPSPA	NS4b 1958–1971	3a	–	6	75	48	–
AAAAEDYPSPAAAA	NS4b 1966–1971 (mp)	3a	–	–	–	11	–
(iii) Substitution by Ala							
FKQKALGLLQTASRA	NS4b 1728–1741	1a	61	48	–	18	–
AAQAALGLLQTASRA	NS4b 1728–1741 (mp)	1a	8	4	–	–	–
PLLDRWKAPDYVPPA	NS5a 2296–2309	3a	15	35	25	44	–
PAADRAAAPDAVAAA	NS5a 2296–2309 (mp)	3a	–	–	25	–	–
EEDEREISVPAEILA	NS5a 2262–2275	1a,1b	77	24	–	–	3
AEDAAEISVPAEILA	NS5a 2262–2275 (mp)	1a	15	15	–	11	23

with the two sets of primers for the 5′ untranslated region of HCV genome described by Larrea *et al*. (1996). The limit of sensitivity of this assay was 1000 copies HCV genome per ml.

Genotypes were determined by RT–PCR of a core region from HCV, followed by hybridization with genotype-specific probes, as described by Viazov *et al*. (1994) and modified by Larrea *et al*. (1996) and Gavier *et al*. (1997).

■ **Statistical analysis.** Differences in peptide recognition by sera were tested by using the Mann–Whitney non-parametric test.

Results

In order to study the immunogenicity of variable regions from HCV proteins and to attempt to differentiate between HCV types and subtypes by ELISA, we compared 187 HCV sequences and selected 543 peptides from 120 regions (Table 1 and Fig. 1 A) that had little or no sequence variability within a given subtype and maximal variability between types and subtypes. These 543 peptides were synthesized and tested by ELISA against the 90 sera from HCV-infected patients from the first panel and against the 35 sera from healthy donors from the fifth panel (Fig. 1 B). An analysis of Fig. 1 shows that peptides were recognized according to one of the following patterns: (i) by none of the sera, 179/543 (33%); (ii) non-specifically, recognized by one or more healthy controls, 171/543 (31·5%); and (iii) specifically, only recognized by the sera of HCV-infected patients, 193/543 (35·5%). Among group (iii), 39/193 (20·2%) were genotype-specific. Moreover, of these 39 genotype-specific peptides, five, 12, 14, two and six were specific for genotypes 1a, 1b, 1, 2 and 3, respectively.

To gain an insight into the relative immunogenicity of

Table 3. Effect of polymerization on peptide recognition by sera of patients infected with different HCV genotypes

Region of polyprotein	Genotype of peptide	Polymerization	Recognition by sera (%)				
			1a	1b	2	3	Controls
NS5a 2267–2275	1a	Monomer	7·3	–	–	–	–
		Polymer	19·5	5·3	–	–	–
NS5a 2274–2287	1a	Monomer	2·4	–	–	–	–
		Polymer	4·9	–	–	–	–
NS5a 2350–2363	1a	Monomer	19·5	–	–	–	2·8
		Polymer	24·4	–	–	–	–
NS4b 1744–1757	1b	Monomer	–	19·7	–	–	–
		Polymer	–	20·4	–	–	–
NS3 1086–1099	1b	Monomer	2·4	3·8	–	2·2	–
		Polymer	2·4	4·5	–	–	–
NS5a 2121–2134	1b	Monomer	–	3·8	–	–	–
		Polymer	–	5·3	–	–	–

–, Not detected.

HCV proteins from genotypes 1a, 1b, 2a, 2b and 3, we calculated the mean recognition of peptides of a given genotype by the sera of patients infected with various genotypes. Thus, the average recognition of peptides from genotype 1a by the sera of patients infected with genotype 1a was calculated by adding the percentage recognition of all 1a peptides and dividing by their number. In this way, a mean value of 9·3 was obtained (shown at the right side of Fig. 1). All other mean recognition values were calculated in an analogous manner (Fig. 1). The mean recognition of peptides 1a, 1b, and 3 by sera infected with genotypes 1a, 1b and 3, respectively, showed no significant differences. It was found that sera from one genotype recognized peptides from the same genotype but also from other genotypes. This cross-recognition was significantly similar between genotypes 1a, 1b and 3. No statistical comparison with peptides from genotypes 2a and 2b could be carried out because only four sera from the first panel were available from this genotype.

From Fig. 1, it is clear that the greatest number of variable regions was located in NS5a and that the most immunogenic regions were found in proteins NS4a, NS4b and NS5a. Some regions were found to be more immunogenic in certain genotypes. Thus, region 25 (NS2b) was more immunogenic in genotype 3 than in genotypes 1 and 2. Region 49 (NS4a) was immunogenic in genotypes 2 and 3 but not in genotype 1. Regions 53 (NS4b), 71 (NS5a) and 77 (NS5a) were immunogenic in genotypes 1 and 3 but not in genotype 2. Region 63 (NS4b) was better recognized by genotypes 2 and 3 than by genotypes 1a and 1b. Region 75 (NS5a) was immunogenic in genotypes 1a, 2 and 3 but not in genotype 1b. Region 78 (NS5a) was much more immunogenic in genotypes 1a and 2 than in genotype 3. Finally, region 81 (NS5a) was more immunogenic in genotypes 1a and 3 than in genotypes 1b and 2.

We investigated a possible correlation between peptide recognition and response to IFN-α therapy. This was done by studying the recognition of all 543 peptides by the sera of 67 patients from the first panel, for whom the outcome of IFN-α therapy was known. No correlation was found between recognition of any of the peptides and the outcome of therapy.

In order to attempt to develop a large number of genotype-specific peptides to assess HCV genotypes by ELISA, we modified 70 peptides that cross-reacted either with the sera of healthy controls (34 peptides) or with sera from other genotypes (36 peptides). Three types of modifications were carried out: (i) displacement of the sequence towards the N or C terminus; (ii) shortening the peptide; and (iii) substitution of some amino acids from the sequence with Ala. These modifications gave a total of 116 peptides. Using the first panel of 90 sera, it was found that these modifications led to the following effects: increase of peptide specificity, loss of recognition or appearance of cross-reactivity with healthy controls. Table 2 shows examples of these results. Of these 116 modified peptides, 21 (18·1%) were rendered genotype-specific.

In order to improve the sensitivity of the genotype-specific peptides, we carried out homopolymerization of the most relevant ones. Thus, we selected the 21 genotype-specific peptides obtained after modification, as well as 22 peptides from the group of 39 genotype-specific peptides that were able to recognize 4% or more of the sera. These 43 peptide monomers afforded 39 homopolymers; yields of the four remaining homopolymers were too low to be used in experimental work. Homopolymerization of peptides had

Table 4. Peptides from HCV proteins used to assess HCV genotypes by ELISA

Peptide homopolymers are indicated in parentheses. Modified peptides are indicated by the suffix (mp). Alanine residues added that are not contained in the wild-type sequence are indicated by A.

Peptide	Amino acid sequence	Region of polyprotein	Genotype	
			Peptide	Assessed
1	AAEISVPAEILAAA	NS5 2267–2275 (mp)	1a	1a
2	ILRKSRRFAQALPVA	NS5a 2274–2287	1a	1a
3	(ILRKSRRFAQALPVA)$_n$	NS5a 2274–2287	1a	1a
4	(RSFGSSSTSGITGDA)$_n$	NS5a 2350–2363	1a	1a
5	SSIVYEAADMIMHTA	E1 210–223	1b	1b
6	YIEQGMQLAEQFKQA	NS4b 1717–1730 (mp)	1b	1b
7	EAAAPVVESKWRALA	NS4b 1744–1757	1b	1b
8	(EAAAPVVESKWRALA)$_n$	NS4b 1744–1757	1b	1b
9	FFTEVDGVRLHRYAA	NS5a 2121–2134	1b	1b
10	(FFTEVDGVRLHRYAA)$_n$	NS5a 2121–2134	1b	1b
11	PLLESWKDPDYVPPA	NS5a 2296–2309	1b	1b
12	AAVPPPRRKRTVVAA	NS5a 2324–2334 (mp)	1b	1b
13	AAAYEATDMIMAAA	E1 214–221 (mp)	1b	1
14	(GSKTLAGPKGPITQA)$_n$	NS2b 1086–1099	1b	1
15	EECSQHLPYIEQGMA	NS4b 1709–1722 (mp)	1a	1
16	HLPYIEQGMMLA	NS4b 1714–1725 (mp)	1a	1
17	HLPYIEQGMQLA	NS4b 1714–1725 (mp)	1b	1
18	(HLPYIEQGMQLA)$_n$	NS4b 1714–1725 (mp)	1b	1
19	(YIEQGMQLAEQFKQA)$_n$	NS4b 1717–1730 (mp)	1b	1
20	AAQAALGLLQTASRA	NS4b 1728–1741 (mp)	1a	1
21	QKALGLLQTASRQA	NS4b 1730–1742 (mp)	1a	1
22	AAALGLLQTASRAA	NS4b 1732–1741 (mp)	1a	1
23	AAALGLLQTATKAA	NS4b 1732–1741 (mp)	1b	1
24	(AAAREDVTFQVAAA)$_n$	NS5a 2142–2149 (mp)	1b	1
25	(TANHDSPDAELIEAA)$_n$	NS5a 2216–2229	1a	1
26	(AAEISVPAEILAAA)$_n$	NS5a 2267–2275 (mp)	1a	1
27	KKRTVVLTESTLSTA	NS5a 2329–2343 (mp)	1a	1
28	TESTVSSALAELATA	NS5a 2336–2349	1b	1
29	ALAELATRSFGSSSA	NS5a 2343–2356	1a	1
30	(ALAELATRSFGSSSA)$_n$	NS5a 2343–2356	1a	1
31	KTFGSSGSSAVDSGA	NS5a 2350–2363	1b	1
32	PDSDAESYSSMPPLA	NS5a 2378–2391	1a	1
33	EECASRAALIEEGQA	NS4a 1709–1722 (mp)	2a	2
34	ASRAALIEEGQRIA	NS4a 1712–1724 (mp)	2a	2
35	ASKAALIEEGQRMA	NS4a 1712–1724 (mp)	2b	2
36	ERSDLEPSIPSEYMA	NS5a 2262–2275	2a	2
37	AAAWITEDYPSPAA	NS4b 1963–1871 (mp)	3a	3
38	QTHRPHPDAELVDAA	NS5a 2216–2229	3a	3
39	FEPLRAETDDVEPSA	NS5a 2256–2269	3a	3
40	TAASRAAGLKDPSFA	NS5b 2720–2733	3b	3

similar effects to those described above for peptide monomers. In Table 3, we give some examples of the effect of homopolymerization.

The 43 peptide monomers and the 39 homopolymers were tested against the five panels of sera described in Methods. This allowed the selection of 40 genotype-specific peptides (Table 4). The recognition of these peptides by the first three panels of HCV-infected sera shows that peptides 1 to 12 were able to differentiate between subtypes 1a and 1b whereas peptides 13 to 40 were only able to differentiate between types 1, 2 and 3 (Table 4 and Fig. 2 A). The sensitivities of assessment of genotypes 1a, 1b, 1, 2 and 3 were 34·1, 48·5, 68·8, 58·3 and 48·9%, respectively, with specificities of 100, 99·1, 97·1, 99·5 and 99% (Fig. 2 B). The overall sensitivity and specificity of the assessment of genotypes 1, 2 and 3 were 64 and 98%, respectively.

We also studied genotype assessment in the 14 sera of the fourth panel, which were coinfected with two HCV genotypes.

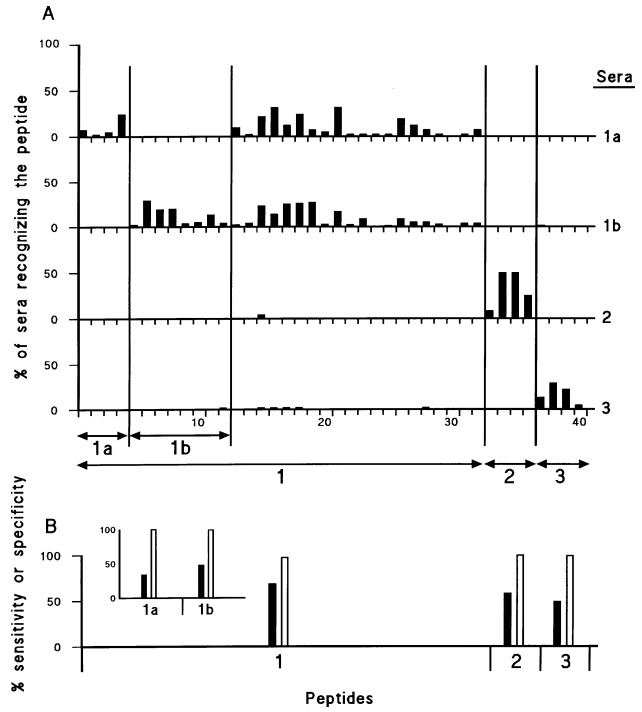

Fig. 2. Recognition of the 40 selected peptides (Table 4) by sera from the first three panels of patients infected with HCV. (A) Peptides 1–4 from genotype 1a, 5–12 from genotype 1b, 13–32 from genotype 1a or 1b, 33–36 from genotype 2 and 37–40 from genotype 3 (indicated by horizontal arrows) were used to assess genotypes 1a, 1b, 1, 2 and 3, respectively, in the sera of patients infected with HCV genotypes 1a, 1b, 2 and 3 (indicated at the right-hand side of the figure). (B) Overall sensitivity (filled bars) and specificity (empty bars) of genotype assessment by the different groups of peptides.

It was found that both genotypes could be assessed correctly in two cases. Only one genotype was assessed in four cases. Neither of the genotypes could be assessed in seven cases. The virus genotype was incorrectly assessed in one case.

Four sera from HCV-infected patients from our serum library, which were PCR negative and could not be genotyped by hybridization with genotype-specific DNA probes, were tested against the 40 peptides from Table 4. This allowed the assessment of the virus genotype in two of the four sera tested.

Discussion

As shown in Fig. 1 and Table 1, a great number of peptides from variable regions of HCV proteins was synthesized in order to identify genotype-specific peptides. No peptides from the hypervariable region of surface protein E2 were synthesized, because sequence variability within this region was so high that no consensus sequence could be attributed to a given genotype.

It was found that the number of peptides from genotypes 1a, 1b, and 3 recognized by the corresponding sera, as well as the mean recognition of peptides by the sera, were not significantly different among genotypes. These findings suggest that the overall immunogenicity of variable regions of HCV proteins from these genotypes is likely to be similar.

Some peptides from a given genotype were not recognized by sera from patients infected with this genotype. However, the sera from patients infected with other genotypes cross-reacted with these peptides and recognized the equivalent peptide from their own genotype with higher intensity. This result may be explained by assuming that a region from a protein may be immunogenic in one genotype but not in others; i.e. peptide 1a from region 49 was not recognized by sera from patients infected with HCV genotype 1a, but was recognized by the sera from patients infected with genotype 3 (Fig. 1B).

It is interesting to note that 27 of the 40 peptides (67·5%) finally selected to assess HCV genotypes were either modified peptides and/or homopolymers, showing that a first selection of peptides based on some amino acid differences was, in many cases, not sufficient to guarantee good specificity and sensitivity. Indeed, from Table 2 and other data not shown, it was found that several epitopes could be found within a sequence of 14–15 amino acids, which may account for the relatively high percentage of cross-reactivity.

Peptide shortening experiments, like the ones shown in Table 2, suggest that B-cell epitopes in the same protein from different HCV genotypes might be located in slightly different positions. This is probably due to preferential antibody recognition of certain topologies in proteins of similar sequence. Thus, since the amino acid sequence affects the fine three-dimensional structure of the protein, certain sequences are likely to be more antigenic than others.

It was found that not all sera from patients infected with the same HCV genotype recognized the same peptides. This might be due to differences in the B-cell repertoire of the patients or to preferential activation of B lymphocytes of different specificities. In this last case, the explanation might be related to the class II molecules of patients. Thus, presentation of certain peptides in conjunction with class II molecules of the patient by the antigen-presenting cells would be responsible for providing T-cell help to a given B lymphocyte specific for a B-cell epitope. Since these helper peptides are class II restricted, some B-cell epitopes, but not others, would be potentiated.

The percentage of peptides that was recognized by at least one serum from the fifth panel of 35 healthy blood donors was 31·5% (Fig. 1). This might be a reflection of the great number of antigens to which we are exposed during our lives, but it may also be related to the high rate of mutation of HCV in response to immune pressure and to the relatively small number of amino acids that define a B-cell epitope (probably around 5–7 amino acids). Moreover, the large panel of peptides that we had to use to cover the different HCV genotypes greatly enhanced the probability of detecting cross-reactivity.

The way a peptide is presented in the ELISA plate may have important effects on the sensitivity and specificity of antibody

detection. In this work, homopolymerization rendered some peptides either non-reactive or less specific but, as shown in Table 3, it also enhanced the sensitivity and/or specificity of others. We used homopolymerization because we had previously shown (Sarobe *et al.*, 1994) that immunization with homopolymers rendered peptides more immunogenic, suggesting better presentation of B-cell epitopes by homopolymers.

The sensitivity of assessment of genotypes 1, 2, 3, 4, 5 and 6 by using synthetic peptides according to the method of Simmonds *et al.* (1993) has been reported to be 89% (Pawlotsky *et al.*, 1997). Using the same method, Cerino *et al.* (1996), Brechot (1996), Agence du Médicaments, France (1996), van Doorn *et al.* (1996), Forns *et al.* (1996) and Martinot-Peignoux *et al.* (1995) reported sensitivities of 51, 80, 82, 83, 85 and 91%, respectively. With the exception of Cerino *et al.* (1996), these values are higher than the 64% sensitivity of genotype assessment of the method reported here to distinguish between genotypes 1, 2 and 3. However, to our knowledge, no serological method has been published that can differentiate between subtypes 1a and 1b, as in the present study.

In spite of the great number of peptides from variable regions of HCV proteins used in the present study, the sensitivity of our method of assessment of genotypes 1, 2 and 3 and subtypes 1a and 1b is still not high enough to match genotype assessment by PCR techniques. However, because of its simplicity, our method could be used to assess the genotype of the virus in an important proportion of sera, and PCR techniques (which are more complicated and require skilled personnel) could then be used if assessment was not possible with peptides. When a serum is HCV-negative by PCR, and consequently cannot be genotyped by PCR techniques, our method can be very useful. Indeed, by using genotype-specific peptides we were able to assess the virus genotype in two of the four sera that were HCV-negative by PCR. We are at present studying constant regions of HCV proteins, in the hope that some of them might be preferentially immunogenic in certain genotypes. Also, we are carrying out competition experiments to eliminate unwanted reactions. Preliminary results show that blocking non-specific cross-reactivity with peptides from the same region but belonging to other genotypes may work for some but not all of the sera tested. This result suggests caution when using this strategy to block cross-reactivity.

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