Virology 269, 313–324 (2000)
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High Prevalence of Hypervariable Region 1-Specific and -Cross-Reactive $CD4^+$ T Cells in HCV-Infected Individuals Responsive to IFN-α Treatment

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Received October 8, 1999; returned to author for revision December 17, 1999; accepted February 2, 2000

The hypervariable region 1 (HVR1) of the putative envelope 2 protein of the hepatitis C virus (HCV) is the most variable part of the whole HCV polyprotein. Anti-HVR1 antibodies have been shown to protect against HCV infection, indicating that this region contains an important neutralization determinant. Recently we and others have demonstrated that HVR1 is also a T cell determinant able to activate helper T cell responses during HCV infection. In order to investigate the role of the immune response against HVR1 during HCV infection we have evaluated the humoral and lymphoproliferative responses to a panel of HVR1 peptides in HCV-infected patients with different outcomes of the disease following interferon-α (IFN-α) treatment. We observed that the frequency of anti-HVR1 T cell responses was significantly higher in patients who recovered after IFN-α therapy than in those who did not, while no differences in the anti-HVR1 antibody reactivities were detected. In addition, by generating HVR1-specific T cell lines and clones we identified human leukocyte-associated antigens DR4 restricted T cell epitopes in the carboxy-terminus of HVR1 and we demonstrated that broadly cross-reactive HVR1 T cells are elicited by HVR1. © 2000 Academic Press

INTRODUCTION

Hepatitis C virus (HCV) is one of the major etiologic agents of chronic hepatitis around the world (Choo *et al.*, 1989). Chronic infections develop in more than 50% of patients, eventually leading to cirrhosis and hepatocellular carcinoma (Alter, 1995). Treatment with interferon-α (IFN-α) is the only available therapy, but long-term benefit is obtained in about 20% of treated cases (Davis *et al.*, 1989). For this reason the development of an anti-HCV vaccine represents an urgent need although a major obstacle to this goal is the high variability of the virus (Martell *et al.*, 1992; Kurosaki *et al.*, 1994; Enomoto and Sato, 1995). Within the HCV polyprotein in the amino-terminus of E2 a hypervariable region (HVR1) consisting of 27 amino acids (aa) has been identified (Weiner *et al.*, 1991). This region is the most variable part of the HCV genome and appears to induce neutralizing antibodies (Zibert *et al.*, 1995; Shimizu *et al.*, 1996; Farci *et al.*, 1994, 1996). Recently some investigators, including our group, have isolated HVR1-specific helper T cells from peripheral blood mononuclear cells (PBMC) of HCV-infected individuals, demonstrating that HVR1 contains T cell epitopes restricted by human leukocyte-associated antigens (HLA-DR) molecules (Frasca *et al.*, 1999; Shirai *et al.*, 1999). Such helper T cells were also detected in mice immunized with HVR1 variants and in both species it could be observed that the anti-HVR1 responses showed a certain degree of cross-reactivity. Interestingly, the presence of antibodies directed to HVR1 was correlated with helper T cell responsiveness to this region in mice and humans, leading to the hypothesis that the cellular response to HVR1 can contribute to the outcome of the infection, helping B cells to produce neutralizing antibodies (Shirai *et al.*, 1999). In order to obtain insight into the role of the anti-HVR1 immune responses during HCV infection, in the present study we evaluated the lymphoproliferative responses and sera reactivity to HVR1 in 44 IFN-α treated patients with different outcomes of the disease. In addition we analyzed the extent of cross-reactivity of the HVR1-specific T cell response by performing a detailed analysis of the specificity of anti-HVR1 T cell lines and clones. Our results demonstrate that (1) HVR1-specific T cell responses are significantly more frequent in patients who succeeded in normalizing the biochemical parameters of disease activity than in those who maintained elevated transaminase values following IFN-α treatment; (2) antibodies to HVR1 are frequently detected in sera of patients and do not correlate with the response to IFN-α; and (3) broadly cross-reactive $CD4^+$ T cells are present in the HVR1 T cell repertoire.

RESULTS

Proliferative responses to HVR1 are frequently detected in PBMC from HCV-infected chronic patients and correlate with long-term response to IFN-α treatment

To investigate the role of anti-HVR1 T cell response during HCV infection we analyzed the proliferative responses of PBMC to synthetic peptides reproducing

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TABLE 1

Clinical and Virological Characteristics of the Patient Population

Patient type[a]	n	Age (years)	Sex (F/M)	Pathology[b] (C/CAH/CPH/CAH-C/nd)	Genotype[c] 1a	1b	2	2a	3	4	nd
LTR	24	50 ± 14	10/14	0/13/6/5/0	1	2	2	6	1	1	11
NR	20	53 ± 11	12/8	1/14/0/3/2	1	10	1	1	4	0	3
R	5	50 ± 20	0/5	0/4/0/1/0	0	1	1	0	1	0	2
RR	4	34 ± 7	3/1	0/2/2/0/0	0	3	0	0	1	0	0
C	15	34 ± 8	8/7								

[a] Patients treated with IFN-α included LTR, long-term responders; NR, nonresponders; R, responders; RR, responders with relapse. C corresponds to healthy controls.

[b] Histological status of liver specimens. C, cirrhosis, CAH, chronic active hepatitis; CPH, chronic persistent hepatitis; nd, not determined.

[c] Genotype was determined according to Simmonds *et al.* (1993).

HVR1 from natural isolates. Due to the wide inter- and intraindividual HVR1 heterogeneity and in the absence of any knowledge of the specific variant of the infecting quasispecies that may have induced a response in the patients analyzed, we used a panel of peptides reproducing HVR1 from natural isolates selected from those available in the databases to represent highly divergent naturally occurring variants. This was done by a multidimensional cluster analysis performed on 234 aligned natural HVR1 sequences (Casari *et al.,* 1995). Forty-three sequences nearly homogeneously distributed over the HVR1 "sequence space" were identified (Puntoriero *et al.,* 1998) and of these, the first 13 sequences were chosen and synthesized as multiple antigenic peptides (MAPs; Pessi *et al.,* 1990). Among these, 8 MAPs were used in proliferation assays of PBMC from IFN-α-treated HCV patients described in Table 1. As control of PBMC proliferation 5 U/ml of human recombinant IL-2 (rIL-2) was added to each culture. We found that PBMC from 16 patients (30%) showed a significant response to at least one of the HVR1 variants and that in about half of these patients reactivity was directed to multiple HVR1 peptide variants (Table 2). Moreover, all 8 variants were immunogenic even if they showed different induction frequencies of T cell proliferation: from 1.8% for variant 291 to 20% for variant 269 (Table 2). Proliferation was specific since none of the 15 HCV sero-negative controls responded to any HVR1 peptide (Table 3).

We next evaluated the frequency of the proliferative responses in the patients studied who were classified according to the outcome of the disease following IFN-α treatment. Twenty-four patients showed a sustained normalization of liver enzymes even after cessation of the therapy (long-term responders: LTR). Five showed normalized aminotransferase (ALT) activity having finished the IFN-α treatment within 6 months (responders: R), 4 showed normalized ALT activity only during IFN-α treatment (responders with relapse: RR), and 20 did not respond to IFN-α (nonresponders: NR). As shown in Table 2, HVR1-specific T cell proliferation was detected in 11 of 24 LTR (45%), 3 of 20 NR (15%), 2 of 5 R (40%), and in none of the RR patients. The difference between LTR and NR was statistically significant ($P < 0.03$), indicating an association between the activation of HVR1-specific T cells and a positive response to IFN-α. A similar trend was also observed in the anti-HVR1 responses in RR and R patients, although in these cases the limited number of patients did not allow a statistically significant correlation to be established. On the contrary, statistical analysis of rIL-2-induced lymphoproliferation (expressed as mean values of sample counts per minute with substraction of the medium control wells) of LTR (24,710 ± 14,466) and NR (26,176 ± 13,881) did not reveal any significant difference.

Anti-HVR1 antibodies detected in sera from HCV-infected chronic patients do not correlate with long-term response to IFN-α treatment

The observed correlation between the T cell response to HVR1 and the outcome of the IFN-α treatment led us to investigate whether any difference in the prevalence of anti-HVR1 antibodies could be detected in the LTR and NR patients. To this aim we assessed the antibody reactivity of sera from the 24 LTR and 20 NR patients to the same panel of eight HVR1 peptides. As reported in Fig. 1 sera from 34 patients (77%) recognized HVR1 sequences and the majority of them were simultaneously reactive to multiple variants. With the exception of variant 275, which was not recognized by any of the sera, and variant 277, which was scored positive by only four sera, all the variant peptides were recognized by antibodies present in a significant fraction of the tested population, ranging from 23 to 48%. In this case, LTR and NR patients showed similar frequencies of reactivity (75 and 80%, respectively; Fig. 1), suggesting that no correlation between antibody response and outcome of the therapy exists. In addition we did not observe any correlation in the specificity of the B and T cell responses against HVR1 peptides in HCV chronically infected patients. In fact in only 5 of 14 patients, whose PBMC proliferated to HVR1 peptides, the simultaneous recognition of the same HVR1

TABLE 2

Anti-HVR1 Lymphoproliferative Responses in IFN-α Treated Patients

Patients[b]	No peptide[c]	HVR1 peptides[a]							
		267	269	275	277	286	290	291	295
LTR									
1	341	2.4	2.6	1.6	1.5	1.4	**3.2**	1.6	2.4
2	227	**4**	2.8	2.1	1.9	2.2	2.4	2.3	2.2
3	317	**9.1**	1.5	0.9	2.2	0.7	1	1	1.2
4	2143	0.7	1.2	0.3	0.6	0.6	1.3	0.7	0.6
5	320	0.7	**7.8**	1	0.6	1	2.5	0.8	1.5
6	158	2	1.8	1.3	1.4	2.3	1.8	1.6	1.4
7	600	**3.1**	**3.8**	1.5	1.3	**8.1**	1.5	0.6	**4**
8	230	**4.4**	1.2	2	1	**4.2**	1.7	1	0.8
9	2341	0.8	0.8	0.6	0.6	0.5	1.1	0.7	0.7
10	1200	0.8	0.1	0.9	0.8	0.7	1	0.8	0.8
11	1167	1.2	1.5	1.1	1.1	1	1.3	1	1.3
12	660	1.1	2.1	1	1.1	0.9	1.2	1	1
13	468	1.1	1.4	1	1.2	0.9	1.5	1.1	1.3
14	1700	1.3	0.6	0.6	0.6	0.6	1	0.8	0.6
15	2693	0.7	1.1	0.5	1	0.3	1.9	1.1	0.6
16	514	1.4	**7.2**	**15**	**15**	**5.2**	1.9	2.5	2.1
17	222	**3.1**	**4.7**	1.2	1.1	1.7	1.3	1.1	2.1
18	4000	1.1	**3**	0.4	1	1	2.5	1	0.7
19	1500	1.2	1	1.1	1.1	0.9	1	0.9	1
20	808	0.9	0.9	1	1	0.8	1.2	0.9	0.9
21	306	2.2	2.9	**3.1**	2.8	2.9	**3.5**	2.2	**3.8**
22	1500	0.5	**9.1**	0.3	1	1.9	**7.6**	1.2	**5.3**
23	2100	1	1.4	0.7	0.6	0.3	1.2	1	0.5
24	120	1	1	1	0.9	1	1	1	0.9
R									
1	282	1.7	1.3	1.5	1	1.5	1.1	1.1	1.3
2	206	1.1	**7**	1	**3.3**	2.1	1.2	1.2	0.9
3	200	1.6	2.5	0.8	0.8	1	2.1	1.5	1
4	349	1.9	**3.1**	1.2	2	1.4	2.1	1.4	1.7
5	542	1.3	1.5	1.5	1.1	1.3	1.1	1	1.6
NR									
1	400	1.8	1	0.9	0.7	1	1	2	1.8
2	1338	1.9	1.8	1.2	1	1.2	1.1	0.8	2.2
3	729	1.6	1.6	1.1	1.5	1.5	1.6	1.6	2
4	2820	0.5	0.8	0.7	0.6	0.5	1	1	0.5
5	1448	0.6	**5.8**	0.4	0.3	0.7	1.9	0.4	0.5
6	849	**3.2**	**3.5**	**3**	2.2	**3.8**	**3.2**	1.3	**3.7**
7	180	**3.3**	**3.8**	1.1	1.1	1.1	1.1	**3.3**	1.1
8	384	2.1	2.1	1.5	1.5	1.8	1.8	1	2
9	365	1.2	1.6	0.6	0.6	1	1	1.2	1.2
10	894	1.4	1.1	1	1	0.9	0.9	1	1
11	354	1.1	0.7	0.5	0.5	0.5	0.7	1	0.8
12	125	0.7	1.2	0.7	0.7	0.6	0.8	1	0.7
13	130	0.9	1	0.8	0.8	0.8	0.9	0.8	1.1
14	1598	1.7	1.8	0.8	0.8	1.1	1.9	0.7	1.3
15	464	1	2.7	1.3	1.3	0.9	1	1	1
16	623	0.4	0.7	1.2	1.2	0.3	0.5	0.3	0.6
17	790	0.7	2	0.6	0.6	0.8	2	1.3	0.6
18	227	1	2.1	0.7	0.7	1	1	1	1.1
19	1476	0.6	1	0.6	0.6	0.7	1	0.7	0.5
20	1319	1	0.8	0.8	0.8	1	0.9	1.2	1
RR									
1	241	1	2	0.8	0.7	1	0.6	0.8	0.7
2	130	1.7	2.7	1.3	0.9	1.8	1.3	1.4	1.2
3	340	0.8	0.8	1	0.8	0.8	0.6	0.8	1.1
4	197	1	1.7	1	0.7	1	1.2	1	2

[a] Values are expressed as SI. Significant SI are in boldface type.
[b] LTR, long-term responder to IFN-α; R, responder to IFN-α; NR, nonresponder to IFN-α; RR, responder with relapse to IFN-α.
[c] Values express cpm.

TABLE 3

Anti-HVR1 Lymphoproliferative Responses in Healthy Individuals

		HVR1 peptide[a]							
	No peptide[b]	267	269	275	277	286	290	291	295
1	133	1.8	1.1	1	1.1	1.3	1.1	1.4	1.5
2	119	1.1	1.1	1.5	1.2	1.1	1.4	1.5	1.1
3	186	0.8	1.7	1.2	1	0.7	0.7	1	0.8
4	215	1.4	2.2	1.8	1.3	1.4	1.4	1.4	1.4
5	145	1.1	1.2	1.2	1.2	1.8	0.8	1.6	0.9
6	228	1	1.3	1	1.1	0.9	1.4	1	0.9
7	260	1.1	1.9	1.1	1.2	1.6	0.7	1	1
8	231	1.3	1.3	1	1.5	1	1.4	1.6	1.1
9	203	0.8	0.9	1.2	0.9	1.4	2	1	0.9
10	505	1.3	1	0.9	1.1	0.7	0.7	0.9	1.2
11	222	1.8	1.3	1.4	1.7	1.1	1.3	1.3	0.8
12	187	1.4	1.9	1.2	1.6	2.3	2.3	1.7	1.9
13	356	2.1	1.9	1.3	1.5	2.2	1.7	1.5	1.9
14	530	1	0.6	0.9	1.2	0.8	2.1	1.2	1
15	418	1	0.8	1	0.8	0.8	1.1	1	1

[a] Values are expressed as SI.
[b] Values express cpm.

sequence by T cells and antibodies could be demonstrated (Table 2 and Fig. 1).

HVR1 sequences elicit multispecific T cell responses

The analysis of the lymphoproliferative responses to HVR1 demonstrated that PBMC from the majority of HCV-infected patients recognized multiple HVR1 peptides. In order to verify whether this was due to the activation of polyclonal T cell responses or of cross-reactive T cells we generated HVR1-specific T cell lines from two responsive patients. PBMC from one patient who showed a long-term response to IFN-α treatment (n22, Table 2) and from one nonresponder patient (n7, Table 2) were expanded and kept in continuous culture by stimulation with single HVR1 variants and IL-2. We obtained three T cell lines specific for peptides 269, 267, and 291 from the nonresponder patient and one T cell line specific for variant 295 from the long-term responder patient. Blocking experiments using the anti-HLA-DR molecule mAb L-243 demonstrated that all the cell lines were restricted by DR molecules (data not shown). Subsequently, the restriction of the HVR1-specific T cell lines was studied using homozygous partially matched, lymphoblastoid cell lines (LCL) as antigen-presenting cells (APC). As shown in Fig. 2, while the T cell lines T2, T16, and T20 specific for variants 269, 291, and 267 proliferated in the presence of DRB1*0404$^+$-LCL, the 295-responsive T11 cell line required DRB1*1101$^+$-LCL for correct presentation of the antigen. Experiments using as APC either DRB1*0404- or DRB1*1101-expressing L cells confirmed that the T cell lines T2 and T20 were restricted by the HLA-DRB1*0404 molecule while the T11 T cell line was restricted by HLA-DRB1*1101. In fact, proliferation values of T2 and T20 T cell lines in response to antigen-pulsed DRB1*0404$^+$-L cells were 15,565 $\pm$ 1068 and 16,025 $\pm$ 1213 counts per minute (cpm), respectively, while the values of the same cells in the presence of unpulsed APC were 1724 $\pm$ 25 and 303 $\pm$ 73 cpm. Similarly, proliferation values of T11 cells with DRB1*1101$^+$-APC in the presence or in the absence of the antigen were 17,195 $\pm$ 3924 and 150 $\pm$ 14 cpm, respectively. The degree of cross-reactivity of T cell lines was evaluated by testing their reactivity to a panel of HVR1 variants. As shown in Fig. 3 while T cell lines T2 and T20 proliferated in response to two and three other different HVR1 peptides, respectively, the T cell lines T16 and T11 recognized only the original peptides used for their selection.

To verify whether the differences in the cross-reactivity of HVR1-specific T cells may be accounted for by focusing the responses on different portions of the HVR1 sequence, we mapped the T cell epitopes recognized by T2, T16, and T20 T cell lines in 269, 291, and 266 variants, respectively. To identify the epitope(s) within the 27-aa-long HVR1 sequence responsible for T cell activation, three overlapping peptides covering the C-terminal, N-terminal, and central sequences of variants 269 and 291 and a subpeptide corresponding to the C-terminus of HVR1 variant 266 were synthesized and their stimulatory activity was tested in a proliferation assay. As shown in Fig. 4, T cell lines T2, T16, and T20 always proliferated in the presence of the C-terminal peptides, indicating that HLA-DR4 restricted T cell epitopes are located in the HVR1 sequences between aa 396 and 410. In the same experiment proliferation of T cell line T2 with 10 μg/ml of

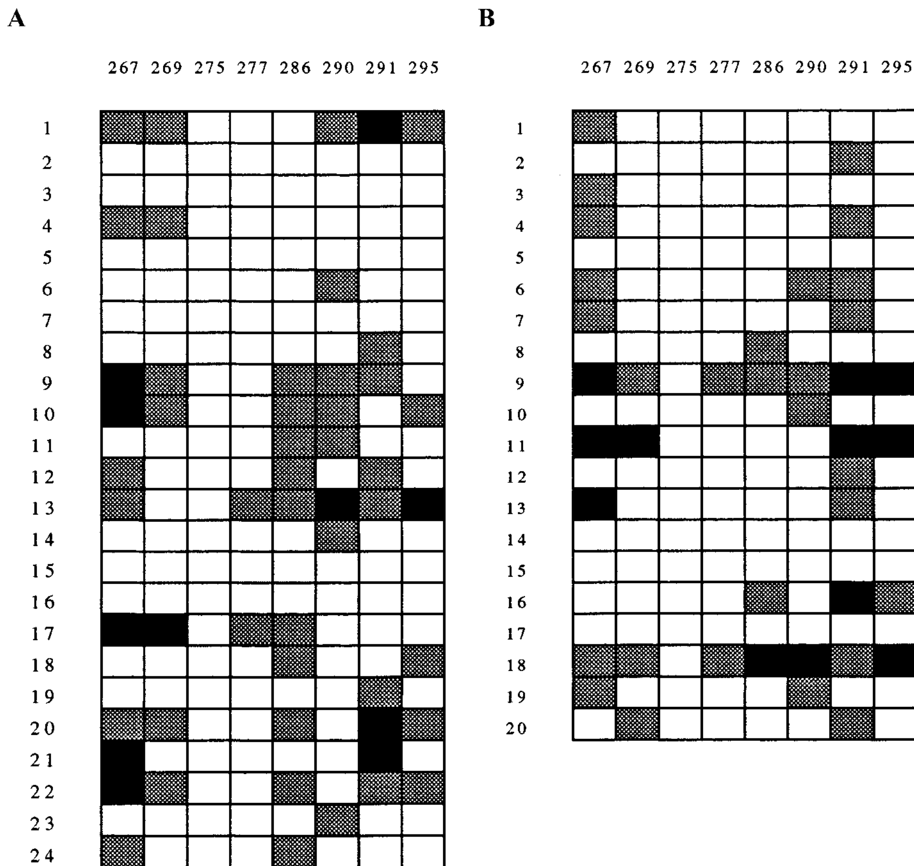

FIG. 1. Reactivity of HVR1 peptides with sera of IFN-α treated patients. Sera from 24 long-term responders (A) and 20 nonresponders (B) were analyzed. The names of HVR1 peptides are indicated at the top of the figure. For each serum, binding of antibodies to the tested HVR1 peptide and to a peptide of unrelated sequence was detected by ELISA. Average values ($A_{405\,nm}$) from two independent experiments have been determined. Results are expressed as the difference between the average value of the tested HVR1 peptides and that of the unrelated controls. Significant reactivities are shown as gray and black boxes. Gray boxes correspond to OD_{405} values ≤0.5, and black boxes correspond to OD_{405} values >0.5. We considered values statistically significant when they differed by more than $3\sigma_{max}$ ($P < 0.003$) from the background signal observed for the unrelated peptide.

peptides corresponding to the N-terminal or central sequence of variant 269 (ATYTTGGSAAKTA, SAAKTAHRLASFF) was 3555 ± 163 and 500 ± 78 cpm, respectively, while proliferation of T cell line T16 with the N-terminal and central peptides of variant 291 (ETHSVGGSAAHTTS, SAAHTTSRFTSLF) was 1205 ± 161 and 1325 ± 9 cpm.

Interestingly we have recently mapped in the corresponding region of variant 295 the T cell epitopes recognized by T cell clones derived from the T11 T cell line (Frasca *et al.,* 1999) and we could confirm the same specificity for the T11 T cell line (data not shown). Thus it seems that the carboxy-terminal region of HVR1 is endowed with helper T cell epitopes.

Clonal HVR1-specific T cells are broadly cross-reactive

We next investigated the HLA restriction of cross-reactive HVR1 variants and their potency in stimulating T cell lines T2 and T20. Restriction analysis using homozygous EBV transformed B cells (EBV-B) expressing the HLA class II DRB1*0404 molecules (Fig. 5A) pulsed with the original and the cross-reactive peptides demonstrate that both T2 and T20 T cell lines use the same restriction element to recognize both the original and the cross-reactive peptides. To compare the potency of cross-reactive variants, the proliferation of cell lines T2 and T20 in response to decreasing concentrations of HVR1 peptides was assessed. The dose–response curve in Fig. 5B shows that at the higher concentration (10 μg/ml) the original and cross-reactive peptides induced similar proliferative responses in both T cell lines, while a 10-fold dilution of peptides 291 and 269 strongly decreased or abolished proliferation of T cell lines T20 and T2, respectively. A much weaker dose–response effect was observed with all the other peptides. To definitively demonstrate the cross-reactive nature of HVR1 T cell recognition, clones from the line T20 were derived and their ability to recognize multiple variants was tested. The pattern of reactivity of representative clones (C6 and C7) to the original (267) and the cross-reactive (291, 266, 286)

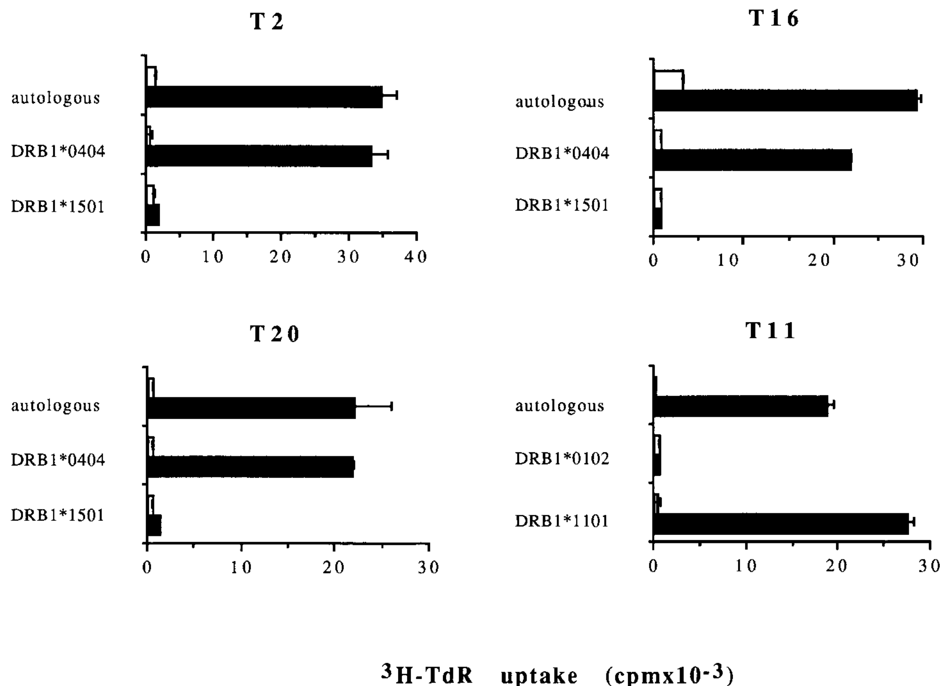

FIG. 2. HLA restriction of HVR1-specific T cell lines. Proliferation of T cell lines specific for HVR1 variants 269 (T2), 291 (T16), 267 (T20), and 295 (T11) was evaluated using as APC partially matched homozygous EBV-B or autologous EBV-B in the presence (black bars) or in the absence (white bars) of 10 μg/ml of HVR1 peptides. Results are expressed as [^{3}H]thymidine incorporation.

HVR1 peptide variants (Fig. 6A) and the dose–response curve of C7 T cell clone (Fig. 6B) closely mirror those observed with the parental cell lines. The confirmation of wide cross-reactivity at the clonal level indicates that a certain degree of TCR flexibility is present in the helper anti-HVR1 T cell repertoire.

DISCUSSION

A high degree of sequence variation in the surface proteins of infectious agents is thought to arise as a consequence of immune selection of escape mutants. Such mutation "hot spots" often contain or are part of neutralizing epitopes (Weiner *et al.,* 1992; Fox *et al.,* 1989; Goudsmith *et al.,* 1989; Javaherian *et al.,* 1989). HVR1 of the putative envelope protein E2 of HCV is the most variable part among all viral proteins (Weiner *et al.,* 1991; Mizushima *et al.,* 1994) and represents another example of this paradigm. In fact, anti-HVR1 antibodies have been shown to prevent HCV infection both *in vitro* and *in vivo,* suggesting that HVR1 is an important determinant for B cells and for the induction of neutralizing antibodies (Farci *et al.,* 1994, 1996; Zibert *et al.,* 1995; Shimizu *et al.,* 1996).

Recently it was shown that HVR1 is also a helper T cell recognition site. Activation of HVR1-specific T cells has been demonstrated during HCV infection and the presence of such cells is associated with the production of antibodies against HVR1 (Shirai *et al.,* 1999). In this study we evaluated the immunogenicity of HVR1 in patients treated with IFN-α and correlated the T and B cell responses with the course of the disease. Moreover by generating HVR1-specific T cell lines and clones we demonstrate that truly broad cross-reactive T cells are present in the HVR1 T cell repertoire.

Using a panel of HVR1 synthetic peptides we detected lymphoproliferative responses against HVR1 in about 30% of the PBMC isolated from patients chronically infected with HCV. Previous studies on the helper T cell response in chronic infection have indicated the core protein as one of the most immunogenic proteins (Ferrari *et al.,* 1994; Hoffmann *et al.,* 1995; Lechmann *et al.,* 1996). Within this protein, immunodominant sequences corresponding to aa 23–42, 66–85, and 131–150 have been identified and the frequency of recognition by PBMC of chronically infected patients was estimated at 10–20% (Hoffmann *et al.,* 1995). Our data indicate that a higher level of prevalence is displayed by epitopes in the HVR1. Moreover, since the peptides used for this analysis were derived from a few, distantly related HVR1 variants, it is possible that by using a larger number of peptides representing additional HCV variants an even higher frequency of T cell reactivity would be observed. In line with this hypothesis are results from a recent study in which, using only four HVR1 variants, it was demonstrated that PBMC from 20% of chronically infected patients recognized HVR1 peptides (Shirai *et al.,* 1999). Thus, it appears that despite its large inter- and intraindividual sequence heterogeneity, HVR1 contains the most commonly recog-

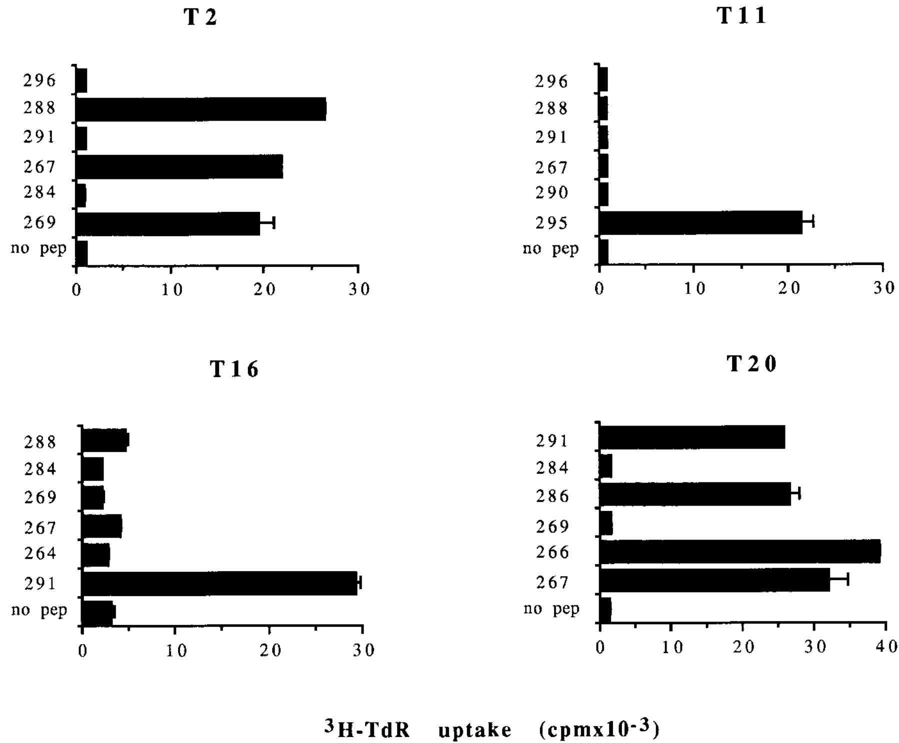

FIG. 3. Proliferative response of specific T cell lines to different HVR1 variants. T cell lines specific for variants 269 (T2), 291 (T16), 295 (T11), and 267 (T20) were incubated with autologous EBV-B in the presence of 10 μg/ml of different HVR1 peptides. Results are expressed as [^{3}H]thymidine incorporation.

nized T epitopes in chronically infected HCV patients described to date. Our results showing that positive responses to HVR1 peptides were significantly higher in IFN-α long-term responder patients than in nonresponder patients indicate that HVR1 may expand a CD4^{+} T cell population with a potential protective role against HCV infection. A higher frequency of T cell responses to E1, core, and NS4 proteins was also detected in sustained responders to IFN-α therapy (Hoffmann *et al.,* 1995; Botarelli *et al.,* 1993; Sarobe *et al.,* 1996; Lasarte *et*

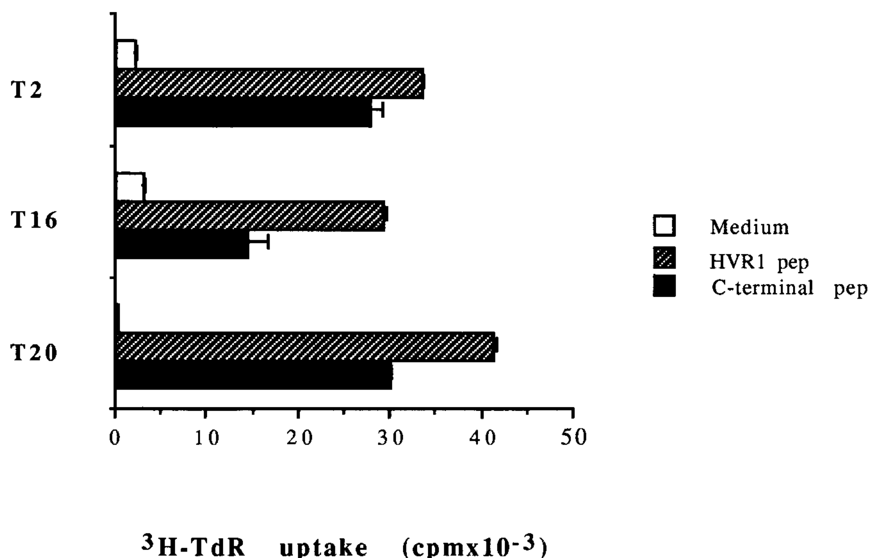

FIG. 4. Mapping of T cell epitopes within the HVR1 sequences. T cell proliferative responses to HVR1 variants 269 (T2), 291 (T16), or 266 (T20) or to peptides encompassing the C-terminus of peptide 269 (RLASFFTVGPKQD), 291 (RFTSLFSPGPQQN), or 266 (TSTLTSLFRPGASQK). Autologous B cell lines pulsed with 10 μg/ml of peptide were used as APC. Results are expressed as [^{3}H]thymidine incorporation.

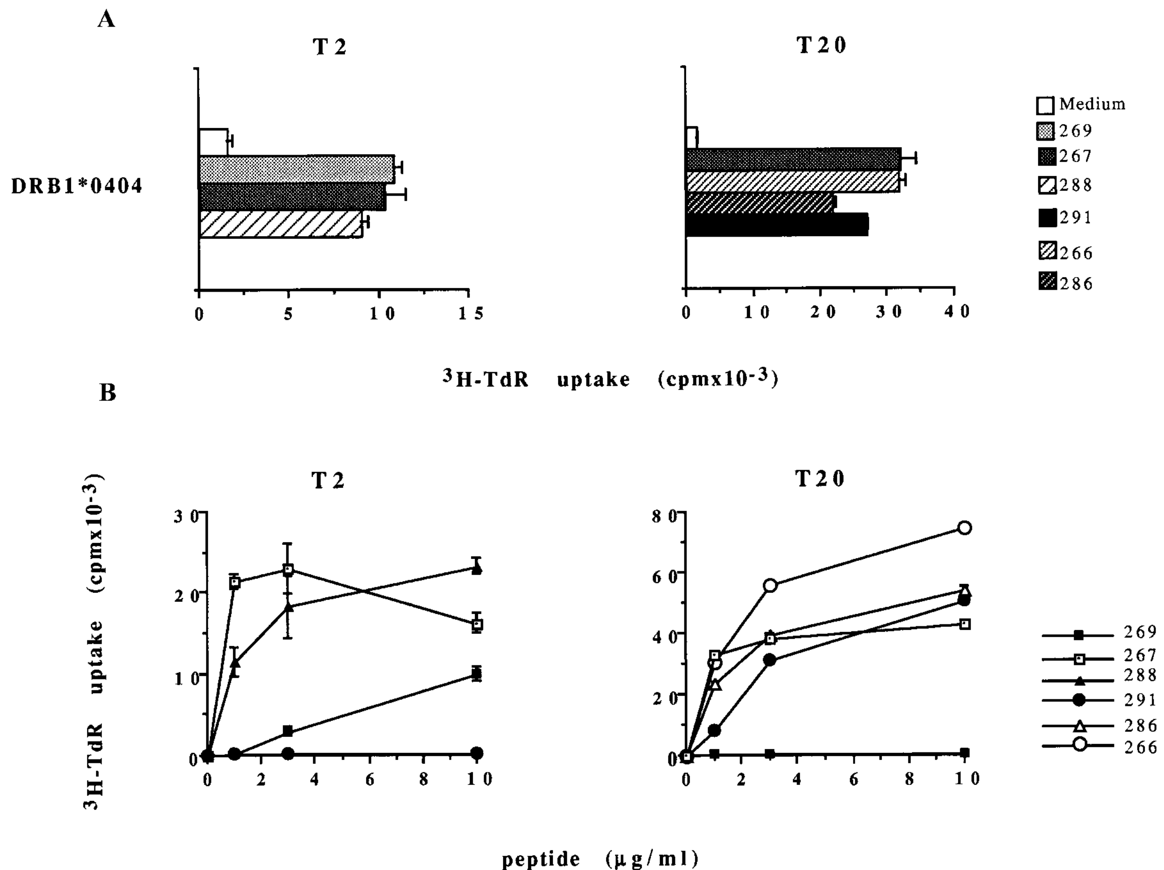

FIG. 5. Analysis of HVR1-specific T-cell cross-reactivity. (A) HLA restriction of cross-reactive peptides. Homozygous EBV-B cells expressing DRB1*0404 were pulsed with 10 μg/ml of HVR1 variants and incubated with T2 and T20 cell lines. (B) Dose–response proliferation of HVR1-specific T cell lines T2 and T20 to multiple HVR1 variants. T cell lines T2 and T20 were incubated with autologous EBV-B and different concentrations of HVR1 variants. Results are expressed as [^{3}H]thymidine incorporation.

al., 1998). Analysis of the T cell responses to core peptides in these patients has recently demonstrated that elimination of HCV is associated with the recognition of multiple core epitopes, but also with a significantly higher frequency of core-specific Th cell precursors (Lasarte *et al.,* 1998). Although our data strengthen the importance of CD4$^+$ T cells in the containment of HCV infection we cannot exclude the possibility that the weaker peripheral blood T cell responses to HVR1 in nonresponders to IFN-α may be caused by persistent sequestration of HCV-specific T cells within the liver of patients with continuing disease.

The humoral immune response to HVR1 has been previously investigated. Zibert *et al.* (1997; see also Lechner *et al.,* 1998) suggested that HVR1-specific antibodies present in an early phase of infection are associated with an acute self-limiting course of the disease. In chronically infected patients anti-HVR1 antibodies have been detected and they seem to be directed mainly against the C-terminus of HVR1 (Zibert *et al.,* 1997b). A fraction of these antibodies have been defined as isolate-independent, because they cross-react with apparently unrelated HVR1 sequences and persist in sera of patients although the sequence of HVR1 changes rapidly (Scarselli *et al.,* 1995; Zibert *et al.,* 1997b). In this study we confirm that sera of almost all chronic patients were reactive with HVR1 peptides and we demonstrate that the anti-HVR1 humoral response is independent of the clinical response to IFN-α.

Our evidence that sera from the majority of chronically infected patients were reactive to HVR1 peptides despite the lack of HVR1-specific T cell responses is apparently at variance with results by Shirai *et al.* (1999), who found that the production of anti-HVR1 antibodies was strictly dependent on the help provided by HVR1-specific T cells. One possible explanation for this discrepancy is that while Shirai *et al.* (1999) analyzed the reactivity of sera against the HVR1 sequence of each patient's own HCV isolate (i.e., isolate-specific antibodies), our analysis was performed with a panel of HVR1 peptides whose amino acid sequences are likely to differ from those of the patients' infecting viruses, allowing for the detection of cross-reactive, isolate-independent antibodies. Thus, we cannot exclude the possibility that differences in the prevalence of isolate-specific antibodies exist in patients with different outcomes of the disease following IFN-α

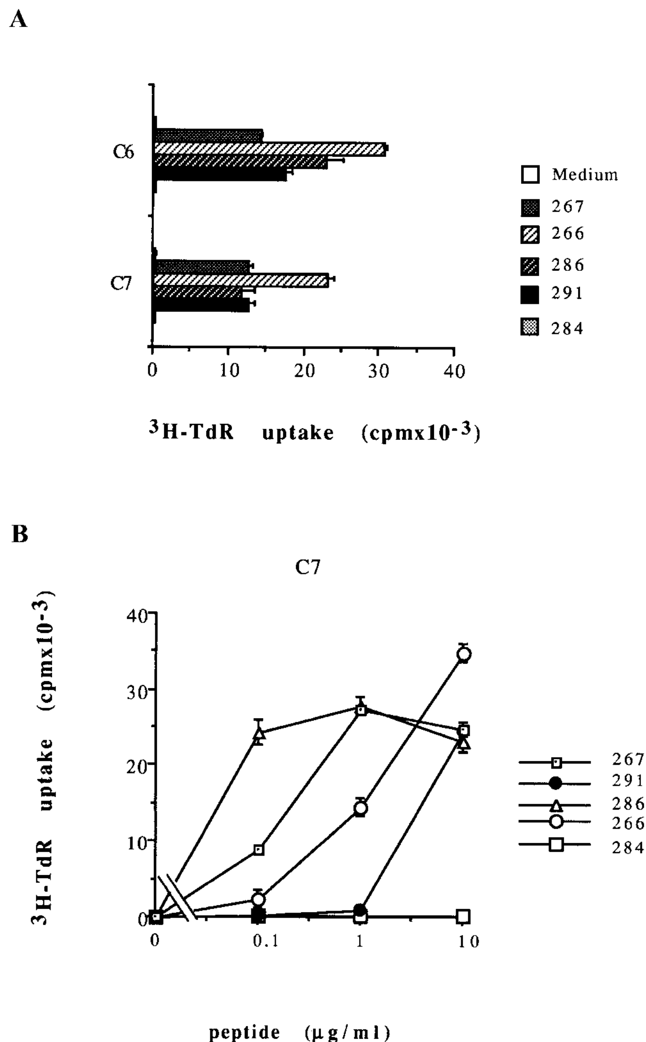

FIG. 6. HVR1-specific T cell clones proliferate to different HVR1 variants. (A) Proliferative responses of clones C6 and C7 to autologous EBV-B pulsed with 10 μg/ml of HVR1 peptide variants. Results are expressed as [^{3}H]thymidine incorporation. (B) Dose–response proliferation of clone C7 to multiple HVR1 peptide variants. Clone C7 was incubated with autologous EBV-B and different concentrations of HVR1 peptide variants. Results are expressed as [^{3}H]thymidine incorporation.

therapy. This point deserves further investigation and would provide helpful information on the mechanism through which HVR1 cellular immune responses play a protective role during HCV infection.

In this study we show that PBMC from the majority of HCV chronically infected patients were reactive with multiple HVR1 peptides. The ability of some HVR1-specific T cell lines and clones to proliferate in response to multiple HVR1 variants indicates that this multireactivity is also due to the activation of cross-reactive T cells. We show that such cells were HLA-DR4 restricted and specific for an epitope located in the carboxy-terminus of the HVR1 sequence (aa 396–410). Interestingly, in the same region we have recently mapped T cell epitopes for HVR1-specific HLA-DR11 restricted T cell clones and identified HVR1 variants acting as T cell receptor antagonists (Frasca *et al.,* 1999). We suggested that the carboxy-terminal sequence of HVR1 variants presents a pattern of conserved amino acid residues in which the hydrophobic amino acid at position 399 could represent a primary anchor for peptide binding to multiple DR alleles (Frasca *et al.,* 1999). Our finding that introducing a nonconservative substitution (L to E) at residue 399 completely abolished stimulatory activity by variant 266 (data not shown) gives further support to this hypothesis. These data together with the identification of DR4 restricted T cell epitopes in the C-terminal sequence of HVR1 by Shirai *et al.* (1999) represent further evidence of the immunodominance of this portion of the HVR1. Moreover the capability of multiple HVR1 sequences to stimulate HLA-DR4 restricted T cell lines demonstrates that the maintenance of MHC binding capacity is a property shared by many HVR1 sequences. The finding that HVR1-specific T cells can recognize peptides that carry multiple amino acid substitutions is consistent with previous studies on the cross-reactivity of structurally related and unrelated antigens with the same TCR (Oldstone, 1987; Hagerty and Allen, 1995; Evavold *et al.,* 1995; Bertoletti *et al.,* 1998). However, the evidence that T cell lines recognizing the same peptide–MHC complex (267 peptide–DR4 for T2 and T20 T cell lines; 291 peptide–DR4 for T20 and T16 T cell lines) display different patterns of cross-reactivity may be explained by the existence of a unique spectrum of secondary T cell contacts necessary for the activation of each individual TCR (Evavold *et al.,* 1992, 1995). In conclusion, our data confirm the high level of immunogenicity of HVR1 and suggest that this region can favor the amplification and the maintenance of protective T cells during HCV infection. These data make the study of HVR1 extremely useful for understanding the influence of viral variation on the evolution of protective immunity during HCV infection. Finally the cross-reactive nature of HVR1 recognition could represent a rationale for the development of synthetic HVR1 sequences able to activate highly multispecific T cells.

MATERIALS AND METHODS

Characterization of patients

Table 1 shows the demographic, clinical, and virological characteristics of the 53 patients enrolled in this study. Diagnosis of HCV infection was based on standard clinical parameters and serological assays with the second-generation (c200/c22-3) Ortho HCV ELISA test system (Ortho Diagnostics). In 51 of the 53 patients with chronic HCV infection, liver biopsies were performed. Forty-two had chronic active hepatitis, 8 had chronic persistent hepatitis, and 3 had cirrhosis. The histological status was defined according to conventional classification.

Patients received between 3 and 6 mU of IFN-α on a

3-week basis for periods ranging from 3 to more than 12 months. Among these patients, 24 were considered LTR since they had normal ALT activity for a period over 6 months after the end of IFN-α treatment. The remaining patients included 5 R (normalization of ALT activity, having finished the IFN-α treatment within 6 months), 4 RR (normalization of ALT activity only during IFN-α treatment), and 20 NR (patients who failed to normalize ALT during IFN-α treatment). All blood samples analyzed were drawn following IFN-α treatment. For all patients, except the 5 responders, 20 ml of heparinized blood was taken after at least 6 months from the end of IFN-α therapy. As control the lymphocyte stimulating activity of HVR1 peptides was analyzed in 15 healthy subjects that showed no sign of past or present HCV infection (i.e., they were negative for anti-HCV in second-generation ELISA tests).

HCV genotypes were determined by reverse transcription-nested polymerase chain reaction (PCR) with sets of primers derived from the highly conserved 5′-noncoding region of the HCV genome and classified according to Simmonds *et al.* (1993).

Choice of a representative panel of HVR1 peptides reproducing the HVR1 sequence of natural isolates

Synthetic peptides reproducing natural HVR1 variants that approximately cover the observed sequence variability were generated as described by Puntoriero *et al.* (1998) and synthesized as multiple antigenic peptides (Pessi *et al.,* 1990).

Accession numbers and sequences are as follows: 264, GenBank D12967, QTRTVGGQMGHGVRGLTSLFSAGSARN, bp 46–126; 266, GenBank D00574, HTRVTGGVQGHVTSTLTSLFRPGASQK, bp 1240–1320; 267, GenBank L19383, ETHTSGGSVARAAFGLTSIFSPGAKQN, bp 46–126; 269, GenBank U24616, ATYTTGGSAAKTAHRLASFFTVGPKQD, bp 22–102; 275, PIR PQ0835, STRITGGSMARDVYRFTGFFARGPSQN, aa 6–32; 277, GenBank D10934, NTYVTGGAAARGASGITSLFSRGPSQK, bp 1491–1571; 284, GenBank X79672, NTRVTGGVQSRTTGTFVGLFTPGPSQR, bp 1–81; 286, GenBank D12952, STRVSGGQQGRAAHSLTSLFTLGASQN, bp 46–126; 288, GenBank M84754, STIVSGGTVARTTHSLASLFTQGASQK, bp 1491–1571; 290, GenBank S24080, NTYVTGGSAGRAVAGFAGLLQPGAKQN, bp 46–126; 291, GenBank S35631, ETHSVGGSAAHTTSRFTSLFSPGPQQN, bp 580–660; 295, GenBank D10687, NTHTVGGTEGFATQRLTSLFALGPSQK, bp 1180–1260; 296, GenBank D43651, NTHVTGGVVARNAYRITTFLNPGPAQN, bp 39–119.

ELISA with MAPs

ELISA multiwell plates (Immunoplate Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 1 mg/ml of MAPs in 50 mM $NaHCO_3$ pH 9.6. The plates were washed in PBS/Tween (PBS/0.05% Tween 20) and blocked for 1 h at 37°C in 250 μl/well of ELISA blocking buffer (5% nonfat dry milk, PBS/0.05% Tween 20). Human HCV plasma, diluted 1:50, was incubated for 2 h at room temperature in 100 μl of ELISA blocking buffer containing 2.5 mg/ml of unrelated MAPs. One hundred microliters of preincubated plasma mixture was added to each well and incubated overnight at 4°C. Plates were then washed with PBS/Tween and incubated for 4 h at 4°C in 100 μl/well of goat anti-human IgG (Fc-specific) alkaline phosphatase-conjugated Abs (Sigma Chemical Co., St. Louis, MO; diluted 1:5000 in ELISA blocking buffer). Alkaline phosphatase was revealed by incubation with 100 μl/well of a 1 mg/ml solution of *p*-nitrophenyl phosphate in substrate buffer (10% diethanolamine buffer, 0.5 mM $MgCl_2$, adjusted to pH 9.8 with HCl). Results were recorded as differences between $OD_{405\,nm}$ and $OD_{620\,nm}$ by an automated ELISA reader (Labsystems Multiskan Bichromatic, Helsinki, Finland).

Generation of T cell lines, T cell clones, and EBV-B

The T cell lines were generated by stimulating patient PBMC with 10 μg/ml synthetic peptide for 10 days. Activated T cells were maintained in culture with irradiated autologous PBMC, peptide, and 20 U/ml of human recombinant IL-2 (Boerhinger Mannheim, Mannheim, FRG) and used at least 3 weeks after the first stimulation. HVR1-specific T cell lines were cloned by limiting dilution in Terasaky plates (0.3 cells/well) in the presence of allogenic PBMC, 0.5 μg/ml phytohemagglutinin (Murex, England), and 20 U/ml human recombinant IL-2. The peptide specificity of T cell lines and clones was assessed by using a proliferation assay. Homozygous lymphoblastoid cell lines used as APC were PE117 (DRA*0101, DRB1*0404, DRB4*0101, DQA1*03, DQB1*0302, DPA1*01, and DPB1*0401), MGAR (DRA*0102, DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602, DPA1*01, and DPB1*0401), MZ070782 (DRA*0101, DRB1*0102, DRB6*0101, DQA1*01, DQB1*0501, DPA1*01, and DPB1*0401), and Sweig (DRB1*1101, DQA1*0501, and DQB1*0301).

EBV transformed B cells from patients were generated by incubation of 5×10^6 PBMC with EBV obtained from the Marmoset lymphoblastoid cell line B95-8 as previously described (Del Porto *et al.,* 1994).

DRB1*0404 and DRB1*1101 transfectants were generated by cotransfection of DRA and DRB cDNAs in the DAP.3 subline of murine L cells and they were kindly provided by Dr. G. Lombardi (Hammersmith Hospital, London, UK).

Proliferation assays

PBMC (1×10^5), isolated from freshly heparinized blood, were resuspended in 0.2 ml of RPMI 1640 (Gibco) containing 10% human serum and incubated at 37°C with 5% CO_2 with 10 μg/ml of synthetic peptide.

After 4 days, 1 μCi of [^{3}H]thymidine was added for 18 h before the cells were harvested.

Stimulation indices (SI) were calculated as the ratio of [^{3}H]thymidine incorporation in the presence of antigen in relation to the control. SI $\geq$ 3 were considered to indicate positive proliferative responses. Standard deviations of the mean counts per min of triplicate cultures were consistently below 30%.

The proliferative response of T cell lines and clones was assessed by incubation of 20 $\times$ 10^3 T cells either with 40 $\times$ 10^3 mitomycin-C treated autologous B cell lines or with 30 $\times$ 10^3 mitomycin-C treated L cell transfectants pulsed with synthetic peptides. After 2 days, the cells were pulsed with 1 μCi of [^{3}H]thymidine and incubated for 18 h before the cells were harvested. The results are expressed as the mean counts per min of triplicate determinations. Data are representative of at least three independent experiments.

ACKNOWLEDGMENTS

We thank Gloria Taliani and Marco Artini for the clinical characterization of patients; Stefano Acali and Antonello Pessi for peptide synthesis; Armin Lahm and Anna Tramontano for determining the HVR1 sequences representative of HCV natural variability; Janet Clench for critically reading the manuscript; Laurent Ferradini for helpful suggestions; and Riccardo Cortese for discussions and continuous support during this work. This work was supported by a grant from the National Health Ministry "First Research Project on Viral Hepatitis" by CNR-PF Biotecnologie and by European Economic Community Contract ERB-CHRXCT930257.

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