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Hypervariable Region 1 Variants Act as TCR Antagonists for Hepatitis C Virus-Specific CD4⁺ T Cells¹

Loredana Frasca,²* Paola Del Porto,²* Loretta Tuosto,¹* Barbara Marinari,* Cristiano Scottà,* Maurizio Carbonari,† Alfredo Nicosia,‡ and Enza Piccolella³*

In various human viral infections, the appearance of mutated epitopes displaying TCR antagonistic activity has been correlated with the severity and persistence of infection. In hepatitis C virus (HCV) infection, where the virus persistence has been associated with the rapid and substantial Ag modifications occurring during replication, TCR antagonism has been evidenced in CD8⁺ T cell responses. However, CD4⁺ T cell antagonism may be another important strategy by which HCV eludes a protective response, because sustained Th responses directed against several HCV Ags are associated with a self-limited course of infection. The data reported here represent the first evidence that variants of the hypervariable region (HVR1) of the putative Envelope 2 protein of HCV can act as powerful TCR antagonists for HVR1-specific CD4⁺ T cells isolated from HCV-infected individuals. Using classical antagonism assays, we observed strong inhibition of cellular proliferation and cytokine production when the agonist and the

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"R"ecognition of the the immunogenic peptides (agonists) in complex with MHC by TCR usually leads to T cell activation. However, small changes in the sequence of the agonist can result in the loss of activatory signals and in the inhibition of T cell response (1–3). When copresented with the agonist, these modified peptides, referred to as TCR antagonists, can abolish agonist-induced T cell activation (1). Because of their capacity to specifically suppress the response to a given Ag, these reagents have been considered suitable for immunological manipulation, such as inhibiting autoimmune and alloreactive responses (4–7). TCR antagonists have thus been artificially synthesized to analyze the mechanisms by which they function and to evaluate their capacity to inhibit in vivo the response of autoreactive T cells (4).

It has recently been shown in various human infectious diseases that the appearance of mutated epitopes displaying TCR antagonistic activity correlates with the severity and persistence of infection (7, 8). In fact, natural antagonist variants of hepatitis B virus (9), HIV (10, 11), hepatitis C virus (HCV)² (7, 8, 12), and more recently Plasmodium falciparum Ags have been identified (13). Functional studies have shown that they can inhibit the cytotoxic activity of CD8⁺ T cells (7–13). Worthy of note is that these naturally occurring T cell epitopes antagonize TCR with an efficiency much higher than that of in vitro-produced altered peptide ligands, suggesting that their appearance is regulated by the selective pressure of the immune system. Hence, these natural variants represent a more suitable tool to characterize the mechanisms involved in TCR signaling by peptide-MHC complexes with agonistic or antagonistic properties.

Because of the pivotal role of cytotoxic T cells in the clearance of viruses, antagonism has mostly been studied in relation to the capacity of highly variable viruses to produce mutated epitopes that inhibit CD8⁺ T cell responses. Nevertheless, an increasing body of evidence indicates that CD4⁺ T cells are crucial to prime and maintain virus-specific CTLs (14). Thus TCR antagonism of CD4⁺ T cell functions may be another important strategy by which mutant viruses can elude a protective response. It has recently been shown that HIV-specific vaccine-induced CD4⁺ CTLs isolated from an infected patient could be antagonized by an epitope sequence of the infecting isolate variant strain in vitro (15). Therefore, we wondered whether TCR antagonism of CD4⁺ T cell responses was a phenomenon evoked by highly mutant viruses. To address this issue, we examined the effect of sequence variation of CD4⁺ T cell epitopes in HCV infection.

HCV is a major cause of chronic hepatitis worldwide (16). Similarly to HIV, HCV establishes persistent infection in >50% of the cases, despite a wide array of B and T cell responses being induced (8, 17). The ability of the virus to undergo rapid and substantial Ag modifications during replication is thought to be a major factor in this process. In fact, HCV exists in the bloodstream of infected patients as quasispecies as a result of the combined action of viral mutation and variants selection by the host immune response (18, 19). Within the HCV genome, a hypervariable region (HVR1),
consisting of the N-terminal 27 aa of the putative Envelope 2 protein (E2), has been identified. This region contains both B and T cell epitopes (8, 20–22). We examined the response of CD4+ T cell lines and clones derived from HCV-infected patients to different peptides reproducing HVR1 sequences of many viral isolates. Our results show that powerful TCR antagonism does occur in HVR1-specific CD4+ T cells in the presence of HVR1 variants. In fact, the total inhibition of cytokine secretion as well as T cell proliferation at low antagonist/agonist ratios were observed. The mechanisms involved in TCR antagonism were addressed in terms of the antagonist capacity to inhibit T cell activation at the level of TCR down-regulation and early signal transduction.

Materials and Methods

Peptide synthesis and purification
Sixteen different natural HVR1 variants (384–410) available in the databank were synthesized as multiple antigenic peptides (MAP) (23). These sequences were chosen among a larger set of HVR1 sequences from natural HCV isolates collectively representing most of the observed viral variability, as described by Punturierato et al. (23). Variant numbers, GenBank accession numbers and sequences of these HVR1 MAPs are reported below: 295, D10687, NTHTVGTGEGFATQLTSLFLGFSQK (1180–1260 bp); 291, S35631, ETHSVGGSAAHTTSRTLSFSGPQQN (580–660 bp); 269, U24616, ATYYTGGSAALKHALSFTTVGPKQD (22–102 bp); 266, D00574, HTRVTGGVQVHSTLFLRPGASQK (1240–1320 bp); 293, S70291, QTRTVGAGAARNTYGLTLFTGF KQN (1–81 bp); 268, M62381, ETHVGSSAGRTTALGGSLTP GAKQN (1426–1506 bp); 302, M62381, ETHVTGGSAGRTTALGGSLTP GAKQN (1426–1506 bp); 299, D30613, GHTVHTGKVATYT TQGFTSSFRGQSK (1491–1571 bp); 296, D43651, NHTVGGV VARIABLEITTLFLNPQQN (39–119 bp); 292, S62953, ETHVT GGAATASTTTLKLFPMQAGSN (43–123 bp); 284, X97662, NTRYT GGQOSIRTGTTFVGLTPQSR (1–81 bp); 294, D84872, GTTT VGASVSTYR枋AFMGSMQGAQN (1485–1565 bp); 303, D84874, ETYYIIGAATRTTALGLSFSSGQSK (1488–1568 bp). Two additional sequences, derived from the pedigreed inoculum H77 (24), were also synthesized as MAPs: 304 (H77–1) ETHVTGNGAAARTTALGLVLTP GAKQN (1–81 bp); 305 (H79) ETHVTGGSAGHTAAGSSAFPGPK QKN (1–81 bp).

Linear 18-mer peptides corresponding to the C-terminal region of MAPs 266 and 295 (residues 393–410 of the HCV polypeptide), and the corresponding leucin to glutammic acid substitution mutants were also synthesized (266C, 295C, 266E, and 295E). Control peptides HCV-core-32-49, HCV-core-32-49 corresponding leucin to glutammic acid substitution mutants were also synthesized (266C, 295C, 266E, and 295E). Control peptides HCV-core-32-49, HCV-core-32-49 corresponding leucin to glutammic acid substitution mutants were also synthesized (266C, 295C, 266E, and 295E).

Generation of T cell lines, T cell clones, and B cell lines
The T cell lines used in this study were generated by stimulating PBMC of patients expressing DRB1*0404 and DRB1*1501 and DRB5*0101 were specific for the HVR1 variant 291 and 269, respectively, and restricted by HLA-DRB1*0404. HVR1-specific C1 and C4 T cell clones were obtained from a patient expressing DRB1*1101 and DRB1*0102, and were specific for the HVR1 variant 295. Two T cell clones, F17 and 2.3C9, specific for peptides HA-306-318 and HCV-core-32-49, respectively, and restricted by DRB1*1101 were used for controls. EBV-transformed B cells (B lymphoblastoid cell lines (B-LCLs)) were generated by incubation of 5 × 10^6 PBMC with EBV obtained from the Marmoset lymphoblastoid cell line B95-8 as previously described (25). The IL-2-dependent murine T cell line CTLL-2 (European Collection of Animal Cell Cultures, Salisbury, U.K.) was cultured in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 10 U/ml hrlr-2, and 10% FCS. The cells were cultured in 25-cm² flasks and were subcultured every 3 days. Before use in a proliferation assay, the CTLL-2 cells were washed twice and cultured overnight in normal culture medium, but without added hrlr-2.

Isolation of RNA and RT-PCR
The mRNA from T cell clones (2.5 × 10^6) was isolated by QuickPrep Micro mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized from oligo(dT)-primed RNA in 20 μl of reverse transcription buffer and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Rockville, MD). The Vβ usage of T cell clones was determined by PCR amplification using 24 Vβ subfamily-specific primers and the Cβ primer 5′-CCGGCTGTCCTCTAGGGCTGCGG3′ as described by Genevée et al. (26). Thirty cycles of denaturation (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 30 s) were conducted in the automated DNA thermal cycler GeneAmp PCR System 2400 (Perkin-Elmer Cetus, Norwalk, CT). PCR products were size fractionated by agarose electrophoresis.

Proliferation assays
Proliferative response of T cell lines and clones was assessed by incubation of 1–2 × 10^6 T cells with 4 × 10^6 mitomycin C (Mit-C)-treated autologous B cell lines pulsed with different concentration of synthetic peptides (usually between 1 and 30 μg/ml). After 2 days, the cells were pulsed with 1 μCi of [ ^3H]thymidine and incubated for 18 h before cell harvesting. The results are expressed as the mean cpm of triplicate determinations. SD was always <10%. Data are representative of at least three independent experiments.

TCR antagonism assay
To test the HVR1 variant peptides for TCR antagonism, we used a modified version of the method described by De Magistris et al. (1). Briefly, autologous APCs (B-LCLs) were prepulsed overnight at 37°C with different doses of agonist peptide (usually between 10 and 30 μg/ml). The choice of the agonist dose was dictated by the evidence that although T cells proliferated significantly to 1 μg/ml of agonist peptide, optimal proliferation was obtained with concentration ranging between 10 and 30 μg/ml. After washing, the APCs were treated with Mit-C, plated in flat-bottom microplate wells, and the variant peptides were added directly into the wells at various concentrations. After a further 5 h of incubation, T cells were added into the wells and proliferation was measured as described.

Evaluation of agonist and antagonist induced TCR down-regulation
Autologous B-LCLs (2 × 10^5) pulsed overnight with 15–30 μg/ml of either agonist or antagonist peptides and washed to eliminate unbound peptide, were mixed with resting 10^6 CS1 or CS4 T cell clones in round-bottom 96-well plates. In the antagonism experiments, the antagonist peptide and the control peptide were added directly into the assay during incubation of the T cells with the APCs previously pulsed with the agonist. The plates were centrifuged at 1000 rpm for 3 min and cultured at 37°C for either 6 or 18 h. After incubation, the cells were washed with PBS 0.5 mM EDTA to disrupt conjugates. Cells were stained with a FITC-conjugated anti-TCR μβ Ab (WT31; Becton Dickinson, San Diego, CA), a PE-conjugated anti-CD4 Ab (Leu-3a; Becton Dickinson), and a peridinin chlorophyll protein-conjugated anti-CD20 Ab (Leu-16; Becton Dickinson) to discriminate between B and T cells in the cell population. The relative TCR and CD4 expression was assessed by using flow cytometric analysis.

Measurement of IL-2, IL-4, and IFN-γ production
T cells (4 × 10^5 cells/well) were cultured with Mit-C-treated B-LCLs (8 × 10^5 cells/well) pulsed or not with the antigenic preparation. The experiment was performed in flat-bottom microtiter plates as described above for the TCR antagonism assay. After 48 h, culture supernatants were collected and stored at –20°C until used. IL-4 and IFN-γ production by the HVR1-specific T cell clones and lines was measured by ELISA using a quantitative sandwich enzyme immunoassay kit (R&D Systems, Minneapolis, MN). IL-2 production was measured by a biological assay. Briefly, culture supernatants were transferred into 96-well round-bottom microtiter plates as triplicate cultures and the plates stored at –20°C until used. In each well, 3 × 10^3 CTLL-2 cells were added. In each experiment, a standard titration for hrlr-2 was included. After 8 h of incubation, proliferation of
Table 1. Characterization of the HVR1-specific T cell lines and clones

<table>
<thead>
<tr>
<th>T Cell</th>
<th>HLA Restrictiona</th>
<th>HVR1 Specificityb</th>
<th>Vβ Usagec</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS1</td>
<td>DRB1*1101</td>
<td>295</td>
<td>6</td>
</tr>
<tr>
<td>CS4</td>
<td>DRB1*1101</td>
<td>295</td>
<td>19</td>
</tr>
<tr>
<td>T7</td>
<td>DRB1*0404</td>
<td>295</td>
<td>ND</td>
</tr>
<tr>
<td>T2</td>
<td>DRB1*0404</td>
<td>295</td>
<td>ND</td>
</tr>
</tbody>
</table>

a HLA restriction for HVR1 variant recognition was assessed by proliferation assay using HLA partially matched homozygous B-LCLs (see Materials and Methods).

b Name of HVR1 MAPs recognized by the T cell lines and clones (see Materials and Methods).

c Vβ gene expression by the HVR1-specific T cells was assessed by RT-PCR using Vβ family-specific primers (see Materials and Methods).

CTLL-2 was measured as [3H]Tdr incorporation by liquid scintillation spectroscopy. The results are expressed as the mean of triplicate cultures. SEs were routinely <10%.

T cell clone stimulation and immunoblotting

T cell clones (10⁶ cells) were activated for 2 min at 37°C with 3 x 10³ B-LCLs (T:APC ratio 3:1) pulsed overnight with different concentrations of agonist peptide or 100 μg/ml of antagonist peptide in a final volume of 50 μl. At the end of incubation, cells were harvested and lysed for 30 min on ice in 1% Nonidet P-40 lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, and 1 mM EGTA in the presence of inhibitors of proteases and phosphatases: 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM pefabloc-sc, 50 mM NaF, 10 mM Na₄P₂O₇, and 1 mM NaVO₄. For antagonist experiments, APCs were pulsed for 10 h with 30 μg/ml of agonist peptide, washed, and incubated overnight with medium alone or 100 μg/ml of the antagonist peptide or 100 μg/ml of the control peptide HA-306-318. Postnuclear lysates, obtained after centrifugation at 14,000 rpm for 10 min at 4°C, were boiled in SDS sample buffer before gel electrophoresis. After equilibration in transfer buffer (25 mM Tris-base, 192 mM glycine, 20% ethanol), gels were transferred to nitrocellulose membranes. Blots were blocked in PBST 0.1% gelatin. At the end of the incubation, blots were extensively washed, incubated with 1/2000 dilution of peroxidase conjugated goat anti-

Results

Natural variants of HVR1 are able to act as TCR antagonists for HVR1-specific CD4⁺ T cell clones

To identify HVR1 variants that act as TCR antagonists, a panel of HVR1 sequences were tested for their ability to stimulate CS1 and CS4 T cell clones specific for variant 295 (Table I). Six variants of 15 tested with nonimmunogenic activity (Fig. 1, a and b) were chosen and tested as TCR antagonists by using a modified version of the method described by De Magistris et al. (1). The results reported in Fig. 1, c and d, clearly show that variant 266 powerfully inhibited the proliferation of both clones in a dose-dependent manner. It should be underlined that an agonist/antagonist ratio of 1:1 was sufficient to induce a 100% decrease of DNA synthesis in CS1 T cell clone. The variants 268 and 299 also behaved as antagonists, but their effect was weaker and limited to CS1 T cell clone only. To further characterize the HVR1-epitope with antagonist activity, we synthesized two 18-mer peptides encompassing the C-terminal region of the agonist and antagonist variants 295 and 266 (295C and 266C). This choice was determined by previous findings showing that the reactivity of HVR1-specific CD4⁺ T cells was directed toward the C-terminal part of the HVR1 (residues 393–410; data not shown). 266C was used to antagonize the proliferation of CS1 and CS4 clones induced by peptide 295C. Peptides HCV-core-32–49 and HA-306-318, known to bind the restriction allele DRB1*1101 used by CS1 and CS4 T cell clones, were used as controls in similar experiments. The data reported in Fig. 2, a and b clearly demonstrate that the antagonistic effect of variant 266 resided in its C-terminal region. Moreover, the lack of phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY) in PBST 0.1% gelatin. At the end of the incubation, blots were extensively washed, incubated with 1/2000 dilution of peroxidase conjugated goat anti-

FIGURE 1. Natural variants of HVR1 are able to act as TCR antagonists for HVR1-specific CD4⁺ T cell clones. CS1 (a) and CS4 (b) T cell clones (1.5 x 10⁵ cells/well) were cultured in the presence of 4 x 10⁴ cells/well of autologous APCs unpulsed (−) or pulsed with 15 μg/ml of the agonist variant (295) or with 15 μg/ml of six different HVR1 variants in flat-bottom microtiter plates. For the antagonist experiment, autologous APCs pulsed overnight at 37°C with either 15 μg/ml (c) or 30 μg/ml (d) of the agonist variant 295 were plated out (4 x 10⁴ cells/well), and six HVR1-variants were added at increasing concentrations 5 h before adding 1.5 x 10⁴ responding T cells. Proliferation was estimated as described in Materials and Methods. Proliferation of T cell clone CS1 to 15 μg/ml of the agonist variant 295 was 30,587 cpm (c). Proliferation of T cell clone CS4 to 30 μg/ml of agonist variant 295 was 47,176 cpm (d).

Δ cpm x 10⁻³

antagonist peptide concentration (μg/ml)
inhibition by the control peptides ruled out the possibility that the antagonistic effect was due to mere competition for MHC binding between agonist and antagonist peptides. T cell clones 2.3C9 and F17 specific for peptide HCV-core-32-49 and for HA-306-318, respectively, and also restricted by DRB1*1101, were not inhibited by peptide 266C (Fig. 2, a and b). The antagonist activity of variant 266 is restricted to TCRs specific for peptide 295C.

The antagonist peptide 266C inhibits cytokine secretion by HVR1-specific T cell clones

In the mouse system, TCR antagonism can result not only in inhibiting proliferation, but also in blocking of release of both Th1- and Th2-type cytokines by T cells (27). Therefore, we decided to establish whether inhibition of proliferation by the strongest antagonist, peptide 266C, was accompanied by inhibiting cytokine release in both CS1 and CS4 T cell clones. These cells can be classified as Th0 in that they produce IL-4, IL-2, and IFN-γ in response to antigenic stimulation (data not shown). Inhibition of the proliferative response of CS1 and CS4 T cell clones by the antagonist 266C paralleled a proportional decrease of secretion of all three cytokines tested (Fig. 3).
FIGURE 4. The antagonist peptide 266C is unable to mediate TCR down-regulation but inhibits agonist-mediated TCR triggering. Autologous APCs ($2 \times 10^5$), pulsed overnight with 30 $\mu$g/ml of either agonist (295C, ●) or antagonist peptide (266C, ○) were mixed with $10^5$ cells/well of T cell clones in round-bottom 96-well plates and cultured at 37°C for either 6 or 18 h (a and b). In the antagonism experiment (c), CS1 T cell clone was cultured for 18 h in the presence of APCs either unpulsed (--) or pre-pulsed with the agonist (295C) or the antagonist (266C) peptide alone, or pre-pulsed with the agonist and then treated with either the agonist peptide (295C + 266C) or the control peptide HCV-core 32-49 (295C+core). The mean of fluorescence intensity in linearized value of TCR expression control for CS4 T cell clone was 82 at 6 h and 99 at 18 h, while the mean of fluorescence intensity in linearized value of CD4 control expression was 2,055 at 6 h and 2,401 at 18 h. The mean of fluorescence intensity in linearized value of control of TCR expression for CS1 T cell clone was 46.5. Data shown are representative of three separate experiments.

Both agonist and antagonist peptides must be presented by the same APC for inhibition to occur

We next asked whether TCR antagonism mediated by peptide 266C would also occur when the agonist and the antagonist peptides were presented by different APCs. For this purpose APCs were separately pulsed with the same amount of agonist and antagonist peptides and subsequently mixed before incubation with HVR1-specific T cell clones. The results in Fig. 5 show that when the agonist and the antagonist peptides were presented by different APCs, T cell antagonism did not occur. This finding suggests that an antagonist can inhibit TCR triggering only when offered to the responding T cells on the same APC as the agonist.

The antagonist peptide 266C fails to induce early tyrosine phosphorylation events in T cells but interferes with agonist-induced signaling

Several reports have described the effect of TCR engagement by class II-restricted antagonist peptides on the early signaling cascade. However, most of these results were obtained following TCR stimulation by agonist and antagonist peptides separately (30, 31). We thus investigated the effect of the copresentation of agonist and antagonist peptides on the early TCR-induced tyrosine phosphorylation events. As shown in Fig. 5a, TCR engagement by peptide 295C induced the dose-dependent tyrosine phosphorylation of a series of cellular substrates probably representing p95$^{\text{vav}}$, SLP-76, ZAP-70, and the recently cloned p36 LAT. In contrast, the maximal concentration of antagonist peptide 266C failed to increase the level of phosphorylation observed following stimulation of T cells with unpulsed APCs. We next verified whether the antagonist peptide was able to interfere with the agonist-induced tyrosine phosphorylation signals. As shown in Fig. 5b, the antagonist peptide 266C strongly inhibited the tyrosine phosphorylation events induced by the agonist 295C. In particular, tyrosine phosphorylation of proteins likely to be SLP-76 and p36 LAT was mostly affected. In contrast, the copresentation of the control peptide HA-306–318 did not have any significant effect. Altogether, these results suggest that the antagonist peptide 266C interferes with the

TCR and CD4 down-regulation in the HVR1-specific T cell clones. The results reported in Fig. 4, a and b demonstrate that peptide 266C failed to induce either TCR or CD4 down-regulation, confirming that it acts as a pure antagonist. Because antagonist peptides are able to interfere with agonist-induced TCR serial triggering (28, 29), we next verified whether peptide 266C could block TCR down-regulation in response to the agonist peptide 295C. APC previously pulsed with the agonist peptide were pulsed with an equal amount of the antagonist peptide 266C. The agonist/antagonist ratio of 1:1, known to induce complete inhibition of T cell clone proliferation, was used. We found that agonist-induced TCR internalization was completely blocked by the antagonist (Fig. 4c), while the addition of the control peptide HCV-core-32-49 did not affect TCR down-regulation.
agonist-induced T cell activation by blocking the early events of the TCR signaling cascade.

Effect of amino acid substitution at a putative HLA-DR binding motif

A comparison between the sequences of the C-terminal region of both peptides 295C and 266C reveals a total homology between residues 399–403. This led us to speculate that this region might contain crucial residues involved in MHC binding, further sustained by comparison of the carboxyl-terminal sequences of many HVR1 variants (23), indicating that the amino acidic positions at residues 399 and 402 were those highly conserved. In addition, the presence of these residues at these sites is compatible with their possible role as P1 and P4 anchors for binding to DR molecules.

In an attempt to verify the hypothesis that position 399 was the primary anchor, we introduced a nonconservative substitution at this residue (L→E) in both agonist and antagonist peptides (295E and 266E). Substitution of L399 with E gave rise to loss of function of both the agonist and antagonist peptides. In fact, while peptide 295E was unable to induce proliferation of the CS1 T cell clone (Fig. 7a), the antagonist mutant 266E was severely impaired in its ability to block proliferation of this clone (Fig. 7b). Similar results were obtained with the CS4 T cell clone (data not shown). These results suggest that residue 399 may be an important anchor for MHC class II binding.

HVR1 variants serve as powerful TCR antagonists for CD4+ T cell lines derived from a different individual

We also verified the possibility that TCR antagonism was a common phenomenon among HVR1 variants and not only a prerogative of the variants analyzed and CS1 and CS4 T cell clones. Thus, we analyzed the effect of different HVR1 variants by testing the response of HVR1-specific T cell lines derived from a different HCV-infected patient. The use of lines instead of clones would probably reflect a more physiological system in vitro, because T cell responses in vivo are either polyclonal or oligoclonal. To this aim, two lines were obtained from a patient expressing DRB1*0404 and DRB1*1501 and DRB5*0101. These lines, T7 and T2, were specific for the variants 291 and 269, respectively, and were restricted by HLA-DRB1*0404 (Table I). Again, nonstimulatory HVR1 variants were selected and then tested for TCR antagonism in a preliminary screening where the inhibition of IL-2 release and [3H]thymidine incorporation at an agonist/antagonist ratio of 1:1 were determined (data not shown). The results in Fig. 8 clearly show that HVR1 antagonist variants are frequently generated. In fact, both T cell lines were inhibited by at least two HVR1 variants with an efficiency ranging between 60 and 100% of the proliferative response at the agonist/antagonist ratios of 1:2.5 and 1:5. A slightly higher agonist/antagonist ratio was required to achieve a relevant inhibition of the proliferative response with these lines as compared with the T cell clones described above. This was expected in that it is more difficult to inhibit a polyclonal T cell response by TCR antagonism. However, with the T7 T cell line an inhibition ranging from 50 to 70% of the [3H]thymidine incorporation has been found at the agonist/antagonist ratio of 1:1 with variants 293, 296, and 298 (data not shown). As shown in Fig. 8, the three HVR1 variants 293, 296, and 298 acted as powerful TCR antagonists for the T7 T cell line (Fig. 8a), while the other two variants (292 and 304) acted as powerful TCR antagonists for the line T2 (Fig. 8b).

Discussion

The data reported here represent the first evidence that HVR1 variants can act as powerful TCR antagonists for HVR1-specific CD4+ T cells isolated from HCV-infected individuals. We show that these variants interfere with agonist-induced T cell activation by inhibiting cellular proliferation and cytokine production, and block TCR down-regulation with the consequent suppression of early signal transduction events. Finally, we provide evidence that these effects were not due simply to competition for MHC class II-binding between the agonist and the antagonist ligand.

From a panel of 16 HVR1 variants we found that at least one functioned as a potent TCR antagonist for each of the different HVR1-specific CD4+ T cell lines and clones analyzed. This antagonistic effect was always found at a low agonist/antagonist ratio. Such strong antagonistic activity of a natural epitope has not been described before for Th cells, although potent peptide antagonists have been
FIGURE 7. L→E substitution at residue 399 in agonist and antagonist peptides abolishes both immunogenicity and antagonistic effect. CS1 T cell clone was cultured with either agonist peptide (295C, ■) or the L→E substituted peptide (295E, ○) prepulsed APCs (a). The antagonism assay was performed by using APCs prepulsed with 15 μg/ml of agonist peptide 295C (b) in the presence of either peptide antagonist 266C (■) or peptide 266E (□). Proliferation of CS1 T cell clone to 15 μg/ml of the agonist peptide 295C was 28,800 cpm.

It has been shown in the mouse system that engagement of the TCR by antagonistic MHC-peptide complexes results in incomplete intracellular signals characterized by a distinct pattern of ζ-chain phosphorylation and failure to activate associated ZAP-70 kinase (30, 31). In human CD8+ T cells, the ζ-chain was found to be constitutively phosphorylated and the antagonist ligands were unable to induce significant modifications, suggesting that the antagonists did not affect protein tyrosine phosphorylation (39). Our evidence that stimulation with antagonist peptides alone failed to induce TCR down-regulation opened up at least two possibilities: either the antagonist induced an incomplete phosphorylation pattern or it did not induce any phosphorylation event at all. Our demonstration that the antagonist-exposed T cells did not show any early phosphorylation pattern (Fig. 6a) and did not become anergic (data not shown) strongly supports the second

FIGURE 8. A group of HVR1 variants serve as powerful TCR antagonists for T7 and T2 CD4+ T cell lines. Autologous APCs prepulsed overnight at 37°C with 10 μg/ml of either the agonist variant 291 (a) or 269 (b) were used in the antagonist assay. Proliferation of T7 (a) and T2 (b) T cell lines to 10 μg/ml of the agonist variants was 10,190 cpm and 8,000 cpm, respectively.
hypothesis. However, evidence that the antagonist peptides totally abrogated TCR down-regulation mediated by the agonist implied that, although unable to mediate intracellular signal transduction, the antagonist might influence the signals delivered by the agonist. In fact, our results demonstrate that TCR-mediated early signal transduction in human CD4+ T cells was strongly inhibited by the antagonist. This is the first piece of evidence in humans that class II-restricted antagonist peptides inhibit Ag-mediated early signal transduction, as observed in class I-restricted antagonists (39). One possible explanation of these findings is that the antagonist may interfere with the productive clustering of the MHC/agonist complexes and TCRs necessary for complete phosphorylation events to occur (36, 39, 40). Alternatively, the low stability of TCR/agonist complexes (41) may reduce the occupancy of TCRs by their ligands, which is crucial to reaching the time threshold for induction of any intracellular signals (28, 38). The latter hypothesis is supported by recent findings on the importance of the duration of antigenic stimulation in determining the fate of naive and effector T cells (42).

Two important prerequisites for altered peptide ligands to function as TCR antagonists are the maintenance of MHC binding capacity and the presence of conservative mutations at the TCR contact residues; HVR1 variants appear to satisfy both requirements. In fact, the comparative analysis of 234 unique HVR1 sequences extracted from databases showed a pattern of conserved residues important for the binding to DR molecules in 80% of the natural viral variants, in particular at positions 399 and 402 (23). The hydrophobic amino acids present at position 399 may, in fact, represent the primary anchor for binding to multiple DR alleles (32). This assumption is supported by our evidence that introducing a nonconservative substitution at residue 399 dramatically abolished both agonistic and antagonistic activity (Fig. 7). Moreover, the aliphatic amino acids L, I, and M, known to act as P4 anchor for DR4 and DR11 binding, are present at position 402 in the HVR1 peptides used to generate the T cell clones CS1 and CS4 and the T cell lines T7 and T2 as well as in HVR1 variants working as antagonists (43, 44). Because these T cells are restricted precisely by DR11 and DR4, respectively, we can presume that residue 402 represents the secondary anchor (P4). Interestingly, in a recent report, decapetides located in the carboxy-terminal portion of HVR1 were described as binding to the highly expressed HLA-class I molecule HLA-A2, and some of these variants antagonize CTL activity (8). Mutated epitopes that still bind the restriction elements have also been identified in the case of other infective agents such as P. falciparum (13), where TCR antagonism also occurs.

With regard to the possible amino acidic residues responsible for signaling through the TCR, many studies (45–47) suggest that these are usually the 3–5 aa residues that protrude upwards from the peptide sequence bound to MHC molecules. Conservative substitutions at these putative TCR contact sites usually allow altered peptide ligands to maintain interaction with the TCR, but at the same time may interfere with the results of this interaction, converting an agonist into a partial agonist or an antagonist ligand (1, 48). On the other hand, it is possible that amino acid substitution at the MHC anchor residues, which still allows MHC binding, induces subtle changes in the peptide conformation, which in turn influences the residues involved in TCR engagement (49). Because HVR1 variant peptides present conservative substitutions at residues close to the putative P1, P4, and P6 anchors, as well as at the possible MHC binding residues, both conditions are satisfied.

Our data on the antagonistic effect of HVR1 variants support the thesis that high viral variability can suppress a Th type of response, which is of particular importance in HCV infection (50–52). It is well known that vigorous and sustained Th responses directed against several HCV Ags are associated with a self-limited course of infection (50–53). In chronically infected patients, a correlation between CD4+ T cell response and viral clearance after IFN-α treatment has been established (51, 54), and our recent data9 on the immunogenicity of HVR1 sequences are in agreement with these observations. Because HCV exists in bloodstream of infected patients as quasi-species (55), T cell antagonism could influence the priming of a CD4+ T cell response toward HVR1 immunogenic variants, which will be presented together during infection.

Although there is evidence that HCV-specific Abs (56–58) and CD8+ T cells (8, 59) can exert selective pressure for the generation of escape mutants, whether CD4+ T cell responses provide analogous pressure is not evident. However, in HIV infection the variation of CD4+ Th epitopes has been described as an important cause of viral persistance (60). Because CD4+ T cells allow the activation of both B and CD8+ T cell populations and produce the necessary factors needed to support their functional differentiation and survival (14, 61), we can hypothesize that also in HCV infection the antagonism of CD4+ T cell help could favor HCV persistence. Only an accurate analysis of HVR1 variants with agonistic and antagonistic activity arising in infected patients may validate our hypothesis. To this aim, HVR1 sequencing from serum samples of infected individuals is in progress.

Our study lends strong support to the idea that CD4+ T cell antagonism may be a phenomenon common to infection mediated by highly mutant viruses, thus representing an important escape mechanism that may favor chronicity.

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References


T lymphocytes by antagonism of a weak borne by a variant hepatitis C virus can prevent viral attachment. J. Immunol. 158:3051.


