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Spectratyping of TCR Expressed by CTL-Infiltrating Male Antigen (HY)-Disparate Allografts

Sean L. Johnston* and Peter J. Wettstein2*†

Minor histocompatibility Ags (HA) play a prominent role in stimulating allograft rejection and are recognized by CTLs that mediate this process. There is limited information regarding the sequences of minor HA peptides and the diversity of minor HA-specific TCRs. In the case of the male minor HA (HY), a peptide presented by H2Db molecules has been sequenced. We have used spectratyping to study the diversities of Vβ usage and β complementarity-determining region 3 (CDR3) lengths of TCRs expressed by CTLs that infiltrate HY-disparate skin allografts during rejection. Spectratyping of RNA from second- and third-set male allografts on CD4-depleted, female recipients showed a reduction in Vβ usage and β CDR3 length diversity with prominent representation of Vβ8 genes. CDR3 sequences, as a group, were characterized by net negative charges resulting from negatively charged residues at positions 5–6 and 10–11. The effects of in vivo anti-Vβ8 Ab treatment on rejection of second-set male allografts were investigated. This Ab treatment had no effect on allograft rejection time and resulted in increased Vβ7 usage in recipients with complete Vβ8 depletion. More interestingly, the net charges of β CDR3s derived from Vβ8-depleted recipients were altered by the inclusion of positively charged and polar residues at positions 4–6. These results indicate that Vβ-specific T cell depletion has no effect on HY-disparate allograft survival, but it alters Vβ usage and changes the characteristics of β CDR3s that facilitate class I peptide recognition.

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†Abbreviations used in this paper: HA, histocompatibility antigen; CDR3, complementarity-determining region 3; MST, median survival time; RT, reverse transcriptase.
the chosen experimental approach must allow differentiation between the β-chains expressed by these two T cell populations. Considerable information can be gained by amplifying, subcloning, and sequencing TCR subunit-encoding transcripts derived from sites of inflammation/rejection, but this approach is handicapped by the potential for sampling errors. Previous studies have indicated that in vivo populations of Ag-specific T cells are characterized by reduction in diversities of Vβ genes, but also the reversal of the net charges in the CDR3s expressed by bystander cells. In this communication, we present the results of spectratype analysis of CTL-infiltrating second- and third-set HY-incompatible skin grafts. Sequencing of single-copy products revealed a net negative charge in CDR3s of chains carrying Vβ8 subfamily members that were represented prominently, but the removal of Vβ8+ CTLs by Ab-mediated depletion resulted in not only the use of alternative Vβ genes, but also the reversal of the net charges in the CDR3s.

Materials and Methods

**Mice and cell lines**

Female and male C57BL/6ByJ (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the barrier facility in Mayo Clinic Division of Animal Medicine (Rochester, NY). The HY-specific and D8-restricted CTL-10 clone (15) was generously provided by Dr. Derry Roopenian, The Jackson Laboratory.

**Skin grafting**

Orthotopic tail skin grafts were transplanted according to the previously described technique (20). Each recipient of primary allografts to be scored for rejection time received a single autograft and two allografts from female donors. Skin grafts were scored at routine intervals for the condition of epidermal scale pattern, pigment, and hair; rejection was scored when no viable signs were observed for both allografts. Recipients of skin grafts for the purpose of priming for subsequent sets of allografts received two allografts that were scored at routine intervals to confirm rejection. Subsequent sets of skin grafts were then applied with one autograft and two allografts applied to the tail of each recipient.

**In vivo Ab-mediated T cell depletion**

CD4+ T cells were depleted by i.v. injection of GK1.5 mAb (21) (in ascites fluid (1 mg total protein/recipient) 1 day before grafting and every 7 days thereafter (100 μg/recipient) until rejection. A comparable regimen was used for depletion of Vβ8+ T cells with i.v. doses (the day before and 6 days after grafting) of 5 mg (total protein) of ascites fluid containing the F23.1 mAb (22); this amount was shown by flow cytometry to be double the amount required for >95% depletion of Vβ8+ T cells in peripheral blood from 2C transgenic mice (23) (data not shown).

**PCR primers**

Primers were synthesized by Mayo Molecular Biology Core Facility. All primers were purified on the basis of retention of the 5’-protecting group (dimethoxytrityl) to eliminate partially synthesized oligonucleotides. Primers with the 5’-protecting group were purified with oligonucleotide purification columns (Applied Biosystems, Perkin-Elmer, Foster City, CA). Nested Cβ region primers (Table I) were designed to amplify the noncoding CDNA strand and progress up the C region segment, with Nest I being homologous to a region near the 3’ end of the C region and Nest III near the 5’ end. CβSeq corresponds to the first 20 bp of the 5’ end of the Cβ region. The CβSpectra primer is identical in sequence to CβSeq, but is labeled with 6-carboxyfluorescein (6-FAM) (Applied Biosystems) at the 5’ end. Vβ-specific primers are homologous to specific regions of the Vβ segment that distinguish different genes and subfamilies (24) and are described in Table I.

### Table I. Vβ-specific primers

<table>
<thead>
<tr>
<th>Vβ Segment Primer</th>
<th>Sequence (5’ to 3’, Coding Strand)</th>
<th>Length of Product Including Vβ-Terminal Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vβ1</td>
<td>CTG AAT GCC CAG ACA GCT CCA AGC</td>
<td>77</td>
</tr>
<tr>
<td>Vβ2</td>
<td>CAA AGA GGT CAA ATC TCT TCC CGG TG</td>
<td>115</td>
</tr>
<tr>
<td>Vβ3</td>
<td>GCT TCT CAG CAA ATC GAC ATG ACT G</td>
<td>117</td>
</tr>
<tr>
<td>Vβ4</td>
<td>TCT ATG GAC AAT CAG ACT GCC TCA</td>
<td>114</td>
</tr>
<tr>
<td>Vβ5.1</td>
<td>CAT TAT GAT AAA ATG GAG AGA GAT</td>
<td>129</td>
</tr>
<tr>
<td>Vβ5.2</td>
<td>AAG GTG GAG AGA GAC AAA GGA TTC</td>
<td>120</td>
</tr>
<tr>
<td>Vβ5.3</td>
<td>AGA AAG GAA ACC TGC CGT GTT</td>
<td>107</td>
</tr>
<tr>
<td>Vβ6</td>
<td>TCA ATA ACT GAA AAC GAT CTT</td>
<td>129</td>
</tr>
<tr>
<td>Vβ7</td>
<td>TAC GAT GTT GAT AGT ACT GCT ACC AGC G</td>
<td>132</td>
</tr>
<tr>
<td>Vβ8.1</td>
<td>CAT TAC TCA TAT GTC GCT GAC</td>
<td>135</td>
</tr>
<tr>
<td>Vβ8.2</td>
<td>CAT TAT TCA TAT GGT GCT GCC</td>
<td>135</td>
</tr>
<tr>
<td>Vβ8.3</td>
<td>TGC TGG CAA CCT TCG AAT AGG A</td>
<td>121</td>
</tr>
<tr>
<td>Vβ9</td>
<td>ATG ATA AGA TTT TGA ACA GGG A</td>
<td>138</td>
</tr>
<tr>
<td>Vβ10</td>
<td>GCA ACT CAT TGT AAA GCA AAC AG</td>
<td>118</td>
</tr>
<tr>
<td>Vβ11</td>
<td>CAA GCT CCT ATA GAT GAT TCA GGG</td>
<td>123</td>
</tr>
<tr>
<td>Vβ12</td>
<td>AAG TCT CTT ATG GAA GAT GGT GG</td>
<td>123</td>
</tr>
<tr>
<td>Vβ13</td>
<td>TCC TCT ATA ACA GTT GCC CTC G</td>
<td>109</td>
</tr>
<tr>
<td>Vβ14</td>
<td>TGT TGG CCA GGT AGA GTC GGT GCA A</td>
<td>118</td>
</tr>
<tr>
<td>Vβ15</td>
<td>GCA CTT TCT ACT GTG AAC TCA GC</td>
<td>141</td>
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<tr>
<td>Vβ16</td>
<td>GGT AAA GTC ATG GAG AAG TCT AAA C</td>
<td>123</td>
</tr>
<tr>
<td>Vβ17</td>
<td>AGA GAT CAG CTA GAT GGT CCT CG</td>
<td>89</td>
</tr>
<tr>
<td>Vβ18</td>
<td>CAG CGG GCC AAA CCT AAC ATT CTC</td>
<td>76</td>
</tr>
<tr>
<td>Vβ19</td>
<td>CTG CTA AGA AAC CAT GTA CCA</td>
<td>68</td>
</tr>
<tr>
<td>Vβ20</td>
<td>TCT GCA GCC TGG GAA TCA GAA</td>
<td>56</td>
</tr>
</tbody>
</table>
**Vβ amplification and sequencing**

cDNA was reverse transcribed from the CβNest I primer using the Ampli-"x PCR Gen protocol (Applied Biosystems). The lower layer contained 1 μl 10X PCR buffer (Promega Corp., Madison, WI), 2 μl MgCl2 (25 mM), 2 μl of a dNTP mixture (1.25 mM each), 10 pmol CβNest I, 7.5 U (0.25 μl) RNasin ribonuclease inhibitor (Promega Corp.), and 2 μl Moloney murine leukemia virus RT (Life Technologies, Grand Island, NY). Ten nanograms of total RNA that had been extracted directly from graft tissue and CTL-10 cells using the Total RNA Extraction Kit (5–8; Boulder, CO) were added with sufficient sterile water to achieve a final volume of 10 μl. Synthesis of cDNA was performed in a thermal cycler (MJ Research, Watertown, MA) by incubating at 95°C for 5 min and 42°C for 30 min. Five minutes before completion of the final incubation, a wax pellet was added; the tube was incubated at 100°C for 5 min to inactivate the Moloney murine leukemia virus RT, and the tube was cooled to 25°C.

Reaction tubes received an upper layer that consisted of 33.25 μl sterile water, 2 μl MgCl2 (25 mM), 4 μl 10X PCR buffer (Promega Corp.), 10 pmol of Vβ-specific primers in a 0.5 μl vol, and 1.25 U Taq DNA polymerase (0.25 μl; Promega Corp.). The PCR conditions were 94°C, 1 min; 37°C, 1 min; and 72°C, 2 min. After two cycles, the annealing temperature was raised to 42°C. After an additional cycle, the annealing temperature was raised to 55°C, and these conditions were maintained for 35 cycles, followed by a final extension of 8 min.

Further amplification was accomplished using nested PCR reactions. One microliter of the product from the first PCR was transferred to a fresh tube, and a nested PCR reaction was run with the substitution of CβNest II and subsequently CβNest III in a total of 50 μl (with appropriate scaling of components). The PCR conditions were 94°C, 1 min; 60°C, 1 min; and 72°C, 2 min for 35 cycles. PCR products were gel purified (25) before sequencing using the Wizard PCR Purification Kit following the manufacturer’s protocol (Promega Corp.). Sequencing was performed on an ABI200 automated sequencer at Mayo Clinic Molecular Biology Core Facility using CβSeq as the primer. Approximately 250 bp were sequenced for each PCR product.

**Spectratyping**

After nested PCR amplification using CβNest II was completed, 6 μl of the product was transferred to a fresh reaction tube containing 2.15 μl sterile water, 0.6 μl MgCl2 (25 mM), 1 μl 10X PCR buffer (Promega Corp.), 10 pmol Cβ Spectra primer in a 0.05 μl vol, and 0.5 U Taq DNA polymerase (0.1 μl; Promega Corp.). Single-stranded primer extension was accomplished by 20 cycles of PCR under the following conditions: 94°C, 1 min; 60°C, 1 min; and 72°C, 2 min for 35 cycles. PCR products were gel purified (25) before sequencing using the Wizard PCR Purification Kit following the manufacturer’s protocol (Promega Corp.). Sequencing was performed on an ABI200 automated sequencer at Mayo Clinic Molecular Biology Core Facility using CβSeq as the primer. Approximately 250 bp were sequenced for each PCR product.

**Results**

Spectratyping of male allograft-infiltrating CTLs

Infiltration of allografts by recipient T cells expectedly involves the immigration of alloantigen-specific T cells accompanied by nonspecific T cells that are attracted by the inflammatory response. It has been reported that the majority of T cells present at inflammatory sites comprise the latter population that would expectedly be characterized by the diverse Vβ usage and CDR3 length observed in normal T cell populations. However, alloantigen-specific T cells should also be present and that population would be expected to exhibit a relatively reduced level of diversity of Vβ usage and CDR3 length as a result of clonal expansion (16, 17).

Spectratyping was used to amplify β-chain transcripts with Vβ gene-specific primers and separate PCR products by electrophoresis to identify overrepresented Vβ genes and those transcripts with reduced CDR3 length diversity. Total RNA was extracted from HY-disparate skin allografts that were judged to be in the process of rejection. cDNA was reverse transcribed with a Cβ primer, and Vβ gene-specific amplification was accomplished with nested Cβ primers paired with primers specific for individual Vβ genes. The amplified products were gel electrophoresed, and the sizes of products were determined by comparison with molecular standards.

We have hypothesized that multiple, sequential sets of allografts incrementally reduce the diversity of Vβ usage and CDR3 length exhibited by HY-specific CTLs. The decreased time of rejection observed with subsequent allografts should provide an advantage to specific memory T cells such that their frequency should increase in the infiltrating population. If this were the case, spectratyping should reveal ever-decreasing complexity in CDR3 length and Vβ usage and focus attention on important Vβ segments, which can be characterized for both common Vβ gene usage between multiple individuals and CDR3 length restriction.

Three B6 mice were primed with HY-disparate allografts and received GK1.5 treatment to eliminate CD4+ T cells following complete rejection of the primary allografts. CD4+ depletion was performed to restrict spectratype analysis exclusively to CTLs. Second-set grafts were then transplanted to decrease the variability between rejection times (26) and reduce the diversity of TCRs expressed by HY-specific CTLs, which was evident in comparisons between primary and secondary responses in the H4 system (manuscript submitted). Spectratyping was performed on CTLs infiltrating second-set allografts as well as two autografts (Fig. 1). Vβ2-, Vβ3.2-, and Vβ8-specific amplification was observed with almost all recipients with accompanying evidence of CDR3 length restriction. The CDR3 length was calculated as the number of amino acids between the terminal Vβ Cys residue and the Jβ GXXG motif, as proposed previously by Rock et al. (27). No products were obtained from the autograft on mouse 2, suggesting limited, if any, T cell infiltration at the time of graft harvest. However, products were obtained from the autograft on mouse 1, and the profile was similar in Vβ usage to the allograft on the same recipient, but the CDR3 length composition was almost entirely different, suggesting that the CTL infiltrates present in these two grafts were comprised of partially overlapping sets of CTLs. This may not be surprising since the allografts and autograft were adjacent on the tail (~3 mm apart) and CTL trafficking through both grafts may have occurred.

We attempted to directly obtain β-chain sequences from all Vβ5.2- and Vβ8-specific products with limited CDR3 length complexity and other products with single CDR3 lengths. Unsuccessful sequencing attempts, due to the presence of two or more products in an amplification, are indicated by an * in Figure 1. Only a single sequencing attempt was successful. The sequence derived from peak A from mouse 2 was characterized by inclusion of Vβ5.2, a negative charge at CDR3 position 5, a positive charge at position 6, and a neutral Jβ segment (Table II and Fig. 2). The ability to sequence a single Vβ8.2 PCR product with a single-length CDR3, which was the only product obtained from that graft, suggests that progeny of only a single Vβ8.2* CTL clone had infiltrated the graft.

**T cells infiltrating sequential HY allografts**

The evolution of the CTL response toward HY in the same individual and the identification of CDR3 characteristics of anti-HY TCRs were monitored in two additional B6 mice that received primary male grafts followed by second- and third-set male grafts. When second-set allografts were harvested, they were replaced with female syngeneic grafts. The evolving CTL response to HY is shown in Figure 3. Vβ5.2 and Vβ8 were present in the second-
ary responses of both mice, similar to that seen in Figure 1. Additional Vβ genes were observed, suggesting increased common Vβ segment usage in comparison with the response described in Figure 1, but CDR3 length diversity was still generally restricted to three or fewer components in these segments. The autograft control for mouse 5 revealed no amplification of any Vβ subfamilies, suggesting there were limiting numbers of infiltrating T cells (data not shown). The results of spectratyping β-chains following third-set grafts demonstrated that Vβ segment usage was further restricted to Vβ4, Vβ5.2, and Vβ8 genes (Fig. 3). It is also interesting to note that CDR3 lengths seen in the third-set response were often, but not always, present in the corresponding secondary response, suggesting that single TCRs may not gain a selective advantage and dominate subsequent rejection episodes in this minor HA system. However, direct sequencing of individual Vβ segments revealed a single CDR3 sequence in mouse 4 that was

FIGURE 1. Results of spectratype analysis of Vβ-specific RT-PCR products amplified from RNA from CTL-infiltrating second-set male allografts. Fluorescence intensity refers to the level of detectable signal following separation of end-labeled PCR products on an ABI 377 WTR DNA-sequencing apparatus. Products labeled with letter codes yielded single-copy nucleotide sequences, and products labeled with * did not yield single-copy sequences.
present in both second-set (C) and third-set (I) responses (Fig. 2 and Table II). This result strongly suggests that the CTLs expressing this β-chain were involved directly in rejection of both second- and third-set male allografts on this recipient. Discovery of additional conserved sequences was most likely precluded by the presence of two or more components in the PCR products.

β-chain products derived from second- and third-set graft-infiltrating CTLs were sequenced (Table II), and the CDR3 sequences

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Peak</th>
<th>Vβ</th>
<th>CDR3</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>A</td>
<td>V8.2-D-Jβ1.3</td>
<td>CASS G E B T L Y F</td>
<td>TGG GCC GAC AGT GAA GAG GAC ACC TGG TAC TTT</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>8.1 7</td>
<td>CASSE GE B T L Y F</td>
<td>TGG GCC GAC AGT GAA GAG GAC ACC TGG TAC TTT</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>8.1 9</td>
<td>CASG P G N B Y F</td>
<td>TGG GCC GAC AGT GAA GAG GAC ACC TGG TAC TTT</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>8.2 8</td>
<td>CASG B S Q N T L Y F</td>
<td>TGG GCC GAC AGT GAC AGT CAA ACC TGG TAC TTT</td>
</tr>
<tr>
<td>5</td>
<td>E</td>
<td>14 9</td>
<td>CASG L G Y A E Q F</td>
<td>TGG GCC GAC AGT CTA GAC AGG GGC TAT GAG CAG TCG TCG</td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td>4 10</td>
<td>CASG Q E L G B A E T L Y F</td>
<td>TGG GCC GAC AGT CAA GAA CTG GCC GAT GCA GAA ACC TGG TAT TTT</td>
</tr>
<tr>
<td>4</td>
<td>I</td>
<td>8.1 9</td>
<td>CASG B P G N T B Y F</td>
<td>TGG GCC GAC AGT GAA GAG GAC ACC TGG TAC TTT</td>
</tr>
<tr>
<td>5</td>
<td>J</td>
<td>8.1 11</td>
<td>CASG B G W G G Y A E Q F</td>
<td>TGG GCC GAC AGT GGC TGG GGG GCC TAC TAT GCT GAG CAG TCG TCG TTT</td>
</tr>
</tbody>
</table>

FIGURE 2. Amino acid and nucleotide sequences of CDR3s expressed by CTL-infiltrating second- and third-set HY-incompatible skin grafts. The “Peak” column refers to labeled peaks in Figures 1 and 3. Amino acid translations are denoted by bold-faced characters. Negatively charged residues are overstruck, and positively charged residues are underlined. Sequences are grouped according to the number of sets of allografts on B6 recipients. Underlined nucleotide sequences are germline encoded.
are included in Figure 2. Eight of the ten sequences were obtained from \( \text{V}_b^8 \) subfamily PCR products, despite sequencing attempts on multiple non-\( \text{V}_b^8 \) products. The ability to obtain single-copy sequences directly from \( \text{V}_b^8 \) products suggested that 1) the number of different transcripts derived from these allografts was restricted despite apparent CDR3 length diversity within the \( \text{V}_b^8 \) PCR products, and 2) there was selective expansion of CTL-expressing \( \text{V}_b^8 \) genes, presumably due to a specific response to HY rather than nonspecific inflammation. All but one of these sequences contained negative charges at either position 5 or 6, and all sequences contained at least one negative charge at these or other positions. In addition, 80% of \( \text{J}_b \) segments bore a negative charge, which is greater than the 58% usage expected from unbiased \( \text{J}_b \) usage (28). Eighty percent of all sequences contained two or more negative charges, which is distinctly different from characteristics of CDR3s specific for a \( \text{D}^b \) influenza nucleoprotein peptide (8, 9), but similar to H4- and CTT-1-specific \( \beta \) CDR3s (11, 12). Only 30% of \( \beta \) CDR3 sequences carried a positive charge, and two of the three sequences carrying positive charges also contained two negative charges; the ratio of charged residues was 19 negative/3 positive. The other distinguishing characteristic of these CDR3s was the inclusion of a Gly residue at position 8 in 80% of sequences, which may either serve as a spacing component, a pocket for a bulky peptide-derived (or class I-derived) side chain, or chance placement of a preferentially encoded residue due to preferential guanine insertion by terminal deoxyuucleotidyl transferase in J regions.

### Table III. \( \text{V}_b^8 \) depletion by F23.1 Ab treatment has no effect on survival times of second-set male allografts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Mice</th>
<th>Survival Times (days)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F23.1</td>
<td>10</td>
<td>6,7,8,8,9,9,9,11</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>7,7,8,8,9,9,10,11,&gt;11</td>
</tr>
</tbody>
</table>

* Underlines indicate median survival times.

**FIGURE 3.** Results of spectratype analysis of \( \text{V}_b^8 \)-specific RT-PCR products amplified from RNA from CTL-infiltrating second- and third-set male allografts sequentially transplanted to individual B6 mice. Fluorescence intensity refers to the amount of detectable signal following separation of end-labeled PCR products. Products on which nucleotide sequencing was attempted are denoted as in Figure 1.

As the second- and third-set responses toward HY + \( \text{D}^b \) exhibited prominent \( \text{V}_b^8 \) subfamily usage with CDR3 length restriction, we assessed the effects of F23.1 Ab-mediated depletion of \( \text{V}_b^8^+ \) T cells on HY-disparate allograft survival. Ten B6 female recipients that had rejected primary male allografts were injected i.v. (1 day before and 6 days after grafting) with 5 mg total protein from ascites fluid containing the F23.1 mAb; this amount had previously been shown to deplete >95% of peripheral blood T cells from 2C
transgenic mice (23) (data not shown). The F23.1-treated mice and 10 normal B6 females (having rejected primary allografts) were grafted 2 days later with second-set male allografts. There was no significant difference between the median survival times (MST) of F23.1-treated (MST = 8 days; range = 6–11 days) and normal (MST = 8.5 days; range = 7–11 days) recipients (Table III), indicating that Vβ8+ T cells were not required for rejection of second-set male allografts.

Second-set male allografts were placed on eight additional B6 female recipients of primary male allografts and subsequent F23.1 + GK1.5 treatment. These second-set grafts were harvested during acute rejection and analyzed by spectratyping (Fig. 4). Five
of the eight recipients showed no evidence of Vβ8 transcripts, indicating complete Vβ8-specific depletion. Vβ8-specific amplification was observed in three recipients of F23.1 treatment, indicating that Vβ8 depletion was not complete in these recipients (Fig. 4). In two of these failures (mice 8 and 10), only single-length Vβ8.1 products were obtained in comparison with the above-described recipients of second- and third-set allografts, in which at least two Vβ8 subfamily members were observed in four of five recipients. A restricted Vβ gene usage profile was observed in the five Vβ8-depleted mice, with only two to four Vβ subfamilies utilized in any single individual. Products from Vβ2- and Vβ5-specific amplification were identified, which was characteristic of the response of untreated individuals. More interestingly, all depleted mice also utilized Vβ7, which was a prominent component in only one of the previously described, untreated recipients. The Vβ7-specific products had very restricted CDR3 lengths (one or two lengths), and single Vβ7 sequences were obtained from four of five individuals (included in Table II). RNA from autografts on two recipients were spectratyped and no products were obtained (Fig. 4).

The CDR3 sequences acquired from F23.1-treated mice are shown in Figure 5. Contrary to CDR3 sequences derived from allografts on normal female recipients, there appeared to be two groups of CDR3s distinguished by the charges of amino acids at...
Spectratyping of HY-specific TCRs

FIGURE 6. Amino acid and nucleotide sequences of the β CDR3 expressed by the CTL-10 HY-specific clone. The amino acid translation is denoted by bold-faced characters. Negatively charged residues are overlined. Underlined nucleotide sequences are germline encoded.

Discussion

The use of spectratyping for dissection of arrays of diverse TCR β-chains has the potential to facilitate the identification of β-chains that are overrepresented at anatomic sites of T cell effector function. The ability to obtain single-copy sequences from PCR products that includes single Vβ genes with limited diversity in CDR3 length is a strong indication that these sequences are included in TCRs expressed by specific T cells rather than nonspecific, by-stander T cells that would expectedly express TCRs with diverse Vβ usage and CDR3 length (16). In this communication, we describe the results of spectratype analysis of TCR β-chains expressed by CTLs infiltrating HY-disparate allografts. Arrays of Vβ-specific PCR products were characterized by reduced diversity in Vβ gene usage and CDR3 length (16). In fact, Vβ usage was prominent in H4-incompatible allografts where the restriction to dominant Vβ usage was prominent in H4-incompatible allografts with anti-Vβ Ab. This approach has been successful at excluding the retardation of allografts expressing H2L4 that has been shown to be preferentially recognized by Vβ8+ CTLs in vitro (29). Anti-Vβ treatment had no effect on the speed of male allograft rejection, so it was not surprising that new Vβ genes predominated in allografts rejected by these recipients. It appears that Vβ usage effectively excluded Vβ7 in most recipients, in that Vβ7 was only seen in one of five normal female recipients that expressed Vβ8 transcripts. However, when Vβ8+ T cells were effectively depleted, a Vβ7 product with limited length heterogeneity was found in each of five recipients, suggesting that the expression of Vβ7 by male allograft-infiltrating CTLs was dependent on elimination of Vβ8+ T cells.

A surprising result was the change in net charge and charged residue placement in β CDR3s following anti-Vβ8 depletion. β CDR3s of CTL-infiltrating male allografts in normal female recipients were similar to those of H4-specific CTLs in exhibiting a net negative charge (11). This characteristic was common to H4-specific CTLs that were cloned in vitro or harvested with rejecting allografts (11, 12, manuscript submitted). Furthermore, β CDR3s derived from male allografts were characterized by high representation of negatively charged residues at positions 4–6, which differs from H4-specific CDR3s. This specific negative charge placement also occurs in CDR3s from the two Vβ8-expressing CTL clones with sequences CASGDNSAEYLF (14) and CASSDLVEYFF (O. Lantz, J. Ridge, and P. Matzinger, personal observation), which links this characteristic to in vitro derived, HY-specific CTL clones. It is apparent that the majority of these CDR3s linked to Vβ8 genes included the Asp residue at position

<table>
<thead>
<tr>
<th>Beta chain</th>
<th>CDR3 Length</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Vβ7-γ1.3</td>
<td>6</td>
<td>CAS S G O N T L Y F</td>
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Note: The CDR3 sequences are germline encoded.
5 due to a single codon shift in the site of recombination, since all three germline Vβ8 genes include this codon following the CASS element (30). However, additional, negatively charged amino acids at this and adjacent CDR3 positions appear to have been the result of N nucleotide additions. This net charge and placement of charged residues does not appear to simply be a feature of Dβ-restricted CTLs, since two previously reported panels of influenza nucleoprotein-specific CTLs expressed β CDR3s with limited representation of any charged residues (8, 9).

The charge distribution in β CDR3s could be altered by pretreatment of female recipients with anti-Vβ8 Abs. This treatment reduced the frequency of Vβ8-specific products; those that remained still exhibited the charge distribution that was characteristic of β CDR3s from untreated recipients. However, there was observed to be a considerable increase in β CDR3s with either positively charged or polar amino acids, the latter of which could be expected to be involved in hydrogen ion pairing. This characteristic is shared with the CTL-10 clone that expresses Vβ7 and the CASSGNTLYF CDR3 sequence. Therefore, Vβ8 depletion results in not only the usage of other Vβ genes, but also alters the net charges of the expressed β CDR3s. It should be stressed that this alteration is not complete, in that β CDR3s with negatively charged residues in positions 4 to 6 can still be found with non-Vβ8 genes, such as Vβ2, even after complete Vβ8 depletion.

The basis for the shift in net charge of β CDR3s is not clear at present, but may be related to the peptide(s) recognized by HY-specific CTLs. An HY peptide presented by Dα1 was predicted by identified mimotopes (13) and, more recently, has been identified and sequenced (6). This peptide, WMHHINDLI, was found through its recognition by the CTL-10 clone. As discussed above, the β CDR3 of this clone is similar to those regions derived from male allografts on Vβ8-depleted recipients, in which positively charged or polar amino acids are prevalent in positions 5–6. We have speculated previously that the negatively charged residues in H4-specific β CDR3s are involved in salt bridges with a positively charged residue in the carboxy end of the H4 peptide (11) that we have recently supported through H4 mimotopes that include a positively charged amino acid at position 7 (data not shown).

It is possible that the same interaction or hydrogen bonding facilitates the binding of specific TCRs to the WMHHINDLI peptide. However, CDR3s joined to Vβ8 genes exhibit a strong net negative charge that would not appear compatible with such binding to the carboxy end of this peptide. The recent crystal structures of mouse and human TCRs (31, 32) suggest that β CDR3s are not in positions that allow binding of β CDR3s to the His residues of this peptide. It would appear that either the CDR3s of HY-specific Vβ8 chains are not required for binding, or another peptide may be recognized by Vβ8–CTLs. The former possibility appears unlikely given the marked, net negative charge and concentration of negative charges at positions 5–6 of these β CDR3s that are not observed in other Dα1-restricted TCR (8, 9). Support for the latter possibility comes from the observation that CTLs expressing the published Vβ8 HY-specific TCR (14) do not recognize the WM HHHINDLI peptide (E. Simpson, personal observation). If this were true, it would imply that anti-Vβ8 depletion resulted not only in a change in Vβ usage, but also shifted the response to a second male-specific peptide that is recognized by CTLs that preferentially use the Vβ7 gene and express β CDR3s with characteristies, i.e., positively charged residues, favoring binding to that second peptide. Experiments are in progress to determine whether 1) HY-specific CTL clones expressing Vβ8 and Vβ7 actually recognize different peptides that can be eluted from Dβ molecules and be separated chromatographically, and 2) a change in recognition of these peptides occurs following Vβ8 depletion in vivo. The results of these experiments will expectedly increase our understanding of the mechanisms underlying preferential T cell responses to immunodominant Ags.

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References

appears to correlate primarily with class II MHC antigen-reactivity. *Immunol. Rev.* 74:29.


