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*J Immunol* 1998; 160:3374-3384; ;
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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)3 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. The Journal of Immunology, 1998, 160: 3374–3384.

Minor histocompatibility Ags (HA)3 play a prominent role in stimulating allograft rejection in donor:host combinations that are matched at the MHC. These Ags are presented as peptides by MHC class I and class II molecules, and this presentation is dependent on the specific binding of individual minor HA peptides by MHC molecules (1, 2). Investigations of the specificity of CTL recognition of class I peptide complexes in this system have focused principally on the identification and sequencing of the target peptides. These studies have led to the identification of a single autosomal peptide in humans (3) as well as peptides derived from male-specific Ags in both mice and humans (4–6).

However, there has been limited investigation of the diversity and characteristics of TCRs specific for individual minor HA peptides. Until recently, only limited numbers of CTL clones specific for single HA peptides have been selected so that characterization of these TCRs has been impossible. The vast majority of studies of TCR diversity have centered on T cell responses to exogenous peptides, e.g., cytochrome c (7) and influenza nucleoprotein (8, 9). However, donor minor HA are endogenous Ags that most likely differ from their recipient homologues by limited, but significant, amino acid interchanges; if recipient homologue peptides were to include specific binding motifs for class I molecules (10), it is possible that these recipient peptides are involved in the selection of responding T cells. Therefore, TCRs that recognize these endogenous peptides may not be comparable in diversity and characteristics with TCRs specific for exogenous peptides that may not have comparably related host counterparts. We have recently completed an analysis of TCR α- and β-chains expressed by extensive panels of CTL clones that recognize the H4 and CTT-1 immunodominant minor HA peptides (11, 12). The results of these experiments indicate that specific Vα and/or Vβ genes may be overrepresented in CTL clones specific for single peptides, but there is no clear requirement for expression of individual Vα or Vβ genes. The most distinctive characteristic of these TCRs is the presence of charged residues in β CDR3s; these panels were characterized by net negative charges in the CDR3s with placement of the charges at positions that may bind to specific amino acids in the carboxy ends of class I-bound peptides or the α-helices of the presenting class I molecules (11).

The study and interpretation of diversity of TCR subunits specific for the H4 and CTT-1 peptides are hindered by the fact that the sequences of these peptides are unknown. This lack of sequence data impedes the analysis of the interactions between CDR3s and peptide:class I complexes. The only minor HA peptides identified and sequenced to date in mice are derived from two male-specific (HY) Ags recognized by CTL; one peptide is presented by Kk molecules (5) and the second is presented by Dβ molecules (6, 13). The HY model system should provide the opportunity to analyze the interactions between minor HA peptide:class I complexes and TCR subunits and identify characteristics of each component that facilitate CTL recognition.

Since a number of CTL clones that recognize HY in the context of Dβ molecules have been described previously (14, 15), we chose to investigate the diversity of CTLs involved in HY-disparate skin allograft rejection. Such in vivo analyses are complicated by the complexities of T cell populations that infiltrate graft sites. These populations of graft-infiltrating T cells would be expected to include both Ag-specific and nonspecific bystander T cells so that

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Received for publication August 12, 1997. Accepted for publication December 8, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This research was supported by Grants AI-16072 and CA-15083 from the National Institutes of Health. The nucleotide sequence data reported in this work have been submitted to the GenBank nucleotide sequence database and have been assigned Accession Numbers AF005602-AF005627.

2 Address correspondence and reprint requests to Dr. Peter J. Wettstein, Department of Surgery, Mayo Clinic, Rochester, MN 55905.

3 Abbreviations used in this paper: HA, histocompatibility antigen; CDR3, complementarity-determining region 3; MST, median survival time; RT, reverse transcriptase.

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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 characterized by reduction in diversities of Vβ19 products (16). Accordingly, spectratyping provides a rapid scan of overrepresented Vβ19-specific PCR amplification, followed by electrophoresis of the products to identify overrepresented Vβ genes and the lengths of products that are dependent on CDR3 lengths. In a number of cases, reamplification with Jβ-specific primers restricts the products sufficiently to obtain CDR3 sequences directly from PCR products (16). Accordingly, spectratyping provides a rapid scan of all β-chain transcripts grouped according to the utilized Vβ gene and the lengths of the products. The direct sequencing of PCR products minimizes Taq polymerase errors in comparison with sequencing of cloned PCR products that requires sequencing of clon products from replicate PCRs to confirm sequences.

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 Dα-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 1) were designed to amplify the noncoding Cβ region 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>
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<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 CTT CAG CAA ATA GAC ATG ACT G</td>
<td>117</td>
</tr>
<tr>
<td>Vβ4</td>
<td>CTG ATG GAC ATA 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 AGG GAA ACC TGC CTG 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 GTC GAT AGT GAC G</td>
<td>132</td>
</tr>
<tr>
<td>Vβ8.1</td>
<td>CAT TAC TCA TAT GTC GCT GAC</td>
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</tr>
<tr>
<td>Vβ8.2</td>
<td>CAT TAT TCA TAT GGT GCT GGC</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>
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<tr>
<td>Vβ10</td>
<td>GCA ACT CAT TGT AAA CGA AAC AG</td>
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<tr>
<td>Vβ11</td>
<td>CAA GCC CTT ATA GAT GAT TCA GGG</td>
<td>123</td>
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<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>
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<td>Vβ14</td>
<td>TGT TGG CCA GGT AGA GTC GGT GCA A</td>
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<td>Vβ15</td>
<td>GCA CTT TCT ACT GTG AAC TCA GC</td>
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<td>GGT AAA GTC ATG GAG AAG TCT AAA C</td>
<td>123</td>
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<td>Vβ17</td>
<td>AGA GAT TCT CAG CTA AGT GTT CCT CG</td>
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<tr>
<td>Vβ18</td>
<td>CAG CGG GCC AAA CCT AAG GCC 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- 
flash PCR Gen protocol (Applied Biosystems). The lower layer con- 
tained 1 µl 10× 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 U Molo- 
ney 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′−3′; Boulder, CO) was added with sufficient sterile water to achieve a final volume of 10 µl. Synthesis of cDNA was performed in a thermal cycler (MJ Re- 
search, Watertown, MA) by incubating at 37°C for 25 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 10× PCR buffer (Promega Corp.), 10 

pmol of Vβ-specific primers in a 0.5 µl vol, and 1.25 U Taq DNA poly- 
merase (0.25 µl; Promega Corp.). The PCR conditions were 94°C, 1 min; 
37°C, 1 min; and 72°C, 2 min. Five minutes before the 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 10× PCR buffer (Promega Corp.), 10 pmol of Vβ-specific primers in a 0.5 µl vol, and 1.25 U Taq DNA poly- 
merase (0.25 µl; Promega Corp.). The PCR conditions were 94°C, 1 min; 
37°C, 1 min; and 72°C, 2 min. Five minutes before the 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 10× PCR buffer (Promega Corp.), 10 pmol of Vβ-specific primers in a 0.5 µl vol, and 1.25 U Taq DNA poly- 
merase (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 manufac- 
turer’s protocol (Promega Corp.). Sequencing was performed on an 
ABI200 automated sequencer at Mayo Clinic Molecular Biology Core Fa-

cility 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 10× 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 accom-

plished by 20 cycles of PCR under the following conditions: 94°C, 1 min; 
60°C, 1 min; and 72°C, 2 min. Spectratype analysis was performed by 
mixing 0.75 µl labeled product, 1.25 µl formamide, 0.5 µl gel-loading 
buffer (50 mg/ml blue dextran, 25 mM EDTA), and 0.5 µl Genescan-500 
TAXA internal lane size standards (Perkin-Elmer). This solution was 
heated denatured for 3 min at 95°C, and 1.8 µl was loaded into each lane of 
a 4.75% polyacrylamide (acrylamide/bis ratio of 19:1), 6 M urea denatur- 

ing gel. The gel had a well-to-read length of 36 cm, used 0.22-mm spacers, 
and was run in an Applied Biosystems model 377 WTR DNA-sequencing 
machine for 2 h at 3 KV. Product length determination was performed using 
the Prism Genescan version 2.0i software package (Applied Biosystems), 
and data analysis was performed using the Genotyper 1.1 software package 
(Applied Biosystems).

**Results**

**Spectratyping of male allograft-infiltrating CTLs**

Infiltration of allografts by recipient T cells is 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 inflam-

matory sites comprise the latter population that would expectedly 
be characterized by the diverse Vβ usage and CDR3 length ob-

served in normal T cell populations. However, alloantigen-specific 
T cells should also be present and that population would be ex-

ected 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 electrophore-
sis 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 electrophoresis, 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 in-

crease in the infiltrating population. This would be the case, specra-
typing 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 be-

tween 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 compari-
sions between primary and secondary responses in the H4 system 
 manuscript submitted). Spectratyping was performed on CTLs in-
filtrating second-set allografts as well as two autografts (Fig. 1).

Vβ2-, Vβ5.2-, and Vβ8-specific amplification was observed with al-

most 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 GXG 
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 re-

cipient, 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 Vβ8.2, 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 indi-

vidual 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 \( \beta \)-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. 

\( \beta \)-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>( V_\beta ) CDR3</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( V_{8.2} )-D-J ( \beta 1.3 )</td>
<td>CASS ( \hat{E} ) G ( \hat{E} ) B T L Y F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( V_{8.4} )-D-J ( \beta 2.3 )</td>
<td>CASS ( \hat{B} ) P G G N T ( \hat{E} ) V F F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( V_{8.1} )-D-J ( \beta 2.4 )</td>
<td>CASS G T G C Q B T L Y F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( V_{8.1} )-D-J ( \beta 1.1 )</td>
<td>CASS G ( \hat{B} ) S Q N T L Y F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( V_{8.2} )-D-J ( \beta 2.1 )</td>
<td>CASS ( \hat{B} ) Y V G Q B T L Y F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( V_{8.1} )-D-J ( \beta 1.1 )</td>
<td>CASS ( \hat{B} ) G W G G Y A E Q F F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( V_{8.2} )-D-J ( \beta 2.1 )</td>
<td>CASS ( \hat{B} ) G W G G Y A E Q F F</td>
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<tr>
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<td></td>
<td>( V_{8.1} )-D-J ( \beta 2.5 )</td>
<td>CASS ( \hat{B} ) P G G N T ( \hat{E} ) V F F</td>
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<td></td>
<td>( V_{8.1} )-D-J ( \beta 2.4 )</td>
<td>CASS ( \hat{B} ) P G G N T ( \hat{E} ) V F F</td>
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<tr>
<td></td>
<td></td>
<td>( V_{8.1} )-D-J ( \beta 1.1 )</td>
<td>CASS ( \hat{B} ) P G G N T ( \hat{E} ) V F F</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 Vb8 subfamily PCR products, despite sequencing attempts on multiple non-Vb8 products. The ability to obtain single-copy sequences directly from Vb8 products suggested that 1) the number of different transcripts derived from these allografts was restricted despite apparent CDR3 length diversity within the Vb8 PCR products, and 2) there was selective expansion of CTL-expressing Vb8 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 Jb segments bore a negative charge, which is greater than the 58% usage expected from unbiased Jb usage (28). Eighty percent of all sequences contained two or more negative charges, which is distinctly different from characteristics of CDR3s specific for a Db1 influenza nucleoprotein peptide (8, 9), but similar to H4- and CTT-1-specific β CDR3s (11, 12). Only 30% of β 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 deoxyurucleotidyl transferase in J regions.

### CTLs infiltrating HY allografts on F23.1-treated recipients

As the second- and third-set responses toward HY + Db exhibited prominent Vb8 subfamily usage with CDR3 length restriction, we assessed the effects of F23.1 Ab-mediated depletion of Vb8+ 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

<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, 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>
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</tbody>
</table>

* Underlines indicate median survival times.
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

<table>
<thead>
<tr>
<th>Mouse Peak</th>
<th>Vβ</th>
<th>CDR3</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 Q</td>
<td>2</td>
<td>9</td>
<td>C S A D P D S S Q D T L Y F</td>
</tr>
<tr>
<td>8 S</td>
<td>2</td>
<td>8</td>
<td>C S A E E W V A E E Q Y F</td>
</tr>
<tr>
<td>9 U</td>
<td>3</td>
<td>9</td>
<td>C A S N R O N S A E T L Y F</td>
</tr>
<tr>
<td>8 T</td>
<td>5</td>
<td>2</td>
<td>C A S L R S S G N T L Y F</td>
</tr>
<tr>
<td>7 R</td>
<td>7</td>
<td>12</td>
<td>C A S F R B R G P S G N T L Y F</td>
</tr>
<tr>
<td>9 V</td>
<td>7</td>
<td>8</td>
<td>C A S R T G Q N T L Y F</td>
</tr>
<tr>
<td>11 X</td>
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<td>6 L</td>
<td>8</td>
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<td>10 W</td>
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<td>9</td>
<td>C A S S R G G S Q N T L Y F</td>
</tr>
<tr>
<td>13 Z</td>
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<tr>
<td>6 N</td>
<td>10</td>
<td>10</td>
<td>C A S S S N N W G Q S D T Q Y F</td>
</tr>
<tr>
<td>6 O</td>
<td>12</td>
<td>10</td>
<td>C A S S S P G T G V A E Q F F</td>
</tr>
<tr>
<td>6 P</td>
<td>14</td>
<td>8</td>
<td>C A W B R G C N S S D Y T P</td>
</tr>
</tbody>
</table>

**FIGURE 5.** Amino acid and nucleotide sequences of CDR3s expressed by CTL-infiltrating HY-incompatible skin grafts on B6 mice following F23.1 treatment. The "Peak" column refers to labeled peaks in Figure 4. Amino acid translations are denoted by bold-faced characters. Negatively charged residues are overstruck, and positively charged residues are underlined. Underlined nucleotide sequences are germline encoded.
Positions 4–6. The first group included Vβ8- and Vβ2-specific products that all included negatively charged groups at these positions, as described in Figure 2 for CDR3s derived from allografts on untreated recipients. Furthermore, two of three of the Vβ8-linked CDR3s, derived from recipients with incomplete Vβ8 depletion, included a Gly residue at position 8. The charge distribution of this group of sequences is contrasted by that of the apparent second group that included Vβ7 genes, in which the majority of sequences included positively charged residues at positions 4–6. The majority of the remaining sequences in this group included polar residues at these positions. It would appear that treatment with anti-Vβ Ab increased the frequency with which CDR3s in the second group were observed. However, complete elimination of Vβ8+ CTLs did not result in a complete shift away from CDR3s with negatively charged residues at positions 4–6, since Vβ2-specific products retained these charges.

As discussed, there have been two HY-specific CTL clones reported to date (14, 15). The α- and β-chains of the first described clone have been sequenced (14); this clone expresses Vβ8.2, and the CDR3 sequence is CASGDNSTLYF, which is similar in negative charge distribution to Vβ8-linked CDR3s expressed by CTLs that infiltrate male allografts. We have now identified the expressed Vβ gene and sequenced the β CDR3 of the CTL-10 clone that was used to identify an HY peptide presented by D b2 (O. Lantz, J. Ridge, and P. Matzinger, personal observation). A disadvantage of spectratyping is, unfortunately, the focus on β-chain transcripts to the exclusion of analysis of Vα gene usage due to the relatively large number of Vα genes. Even if Vα analysis were feasible, it would still be impossible to assess α- and β-chain expression in individual CTLs in vitro, as is possible with analysis of individual CTLs that are cloned in vitro.

The restriction to dominant Vβ8 gene usage following second- and third-set male allograft placement was more pronounced than we have observed previously for H4-incompatible allografts (manuscript submitted). Vβ8 usage was prominent in H4-incompatible grafts, but it did not dominate spectratypes of CTLs infiltrating second- and third-set grafts in which a number of Vβ genes were observed that were not present in HY-incompatible grafts. Therefore, we attempted to suppress rejection of HY-incompatible allografts with anti-Vβ Ab. This approach has been successful at retarding the rejection of allografts expressing H2Ld4 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 Vβ8 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.

Spectratype analysis highlighted the prominence of β-chains utilizing Vβ8 gene segments in CTLs infiltrating second- and third-set male allografts. Although Vβ8 subfamily members are the most frequently expressed Vβ genes in normal mice of C57BL strains (9), the prominence of Vβ8 PCR products and the ability to obtain multiple, single-copy sequences from these products indicated specific overrepresentation of Vβ8 gene usage in these rejecting allografts. In fact, PCR products derived from a second-set allograft on one female B6 recipient were confined to a single Vβ8.2 β-chain, suggesting monoclonality in this individual response. Furthermore, identical β-chains including Vβ8.1 were recovered from PCR products derived from both second- and third-set allografts on an individual recipient. The relatively low probability of sequencing identical β-chains from two different allografts supports the contention that CTLs expressing this β-chain were involved in HY-specific, allograft rejection. The proposed prominence of Vβ8 expression in the in vivo response to HY is supported by the fact that one of the two reported HY-specific CTL clones (14) expresses Vβ8.2, and a second unpublished clone expresses Vβ8.3 (O. Lantz, J. Ridge, and P. Matzinger, personal observation). A disadvantage of spectratyping is, unfortunately, the focus on β-chain transcripts to the exclusion of analysis of Vα gene usage due to the relatively large number of Vα genes. Even if Vα analysis were feasible, it would still be impossible to assess α- and β-chain expression in individual CTLs in vitro, as is possible with analysis of individual CTLs that are cloned in vitro.

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 include 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, bystander 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 lengths. In fact, diversity was reduced to the point in which single-copy sequences could be obtained from PCR products with limited CDR3 length diversity without resorting to reamplification with Vβ-specific primers, as previously described in other spectratype studies of in vivo T cell responses (16, 18). The extent of this reduction is most likely dependent, in part, on recognition of a limited number of HY Ag-associated peptides.
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, additionally, 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 β CDRL3s with limited representation of any charged residues (8, 9).

The charge distribution in β CDRL3s could be altered by pre-treatment of female recipients with anti-Vβ8 Ab. This treatment reduced the frequency of Vβ8-specific products; those that remained still exhibited the charge distribution that was characteristic of β CDRL3s from untreated recipients. However, there was observed to be a considerable increase in β β CDRL3s 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-1 clone that expresses Vβ7 and the CASSGGNTLYF CDR3 sequence. Therefore, Vβ8 depletion results not only in the usage of other Vβ genes, but also alters the net charges of the expressed β CDRL3s. It should be stressed that this alteration is not complete, in that β β CDRL3s 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 CDRL3s is not clear at present, but may be related to the peptide(s) recognized by HY-specific CTLs. An HY peptide presented by D9 was predicted by identified mimotopes (13) and, more recently, has been identified and sequenced (6). This peptide, WMHHNDLII, was found to be a considerable increase in

...immunodominant Ags.

Acknowledgments

We acknowledge the excellent technical assistance of Michael Straubausch and the excellent secretarial assistance of DeAnn Frederixon. We thank Dr. Derry Roopenian, The Jackson Laboratory, for generously providing CTL-10 cells.

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


