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

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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. The Journal of Immunology, 1998, 160: 3374–3384.

Minor histocompatibility Ags (HA) 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 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).

The study and interpretation of diversity of TCR subunits specific for 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).

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
the chosen experimental approach must allow differentiation between the \( \beta \)-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 \( \beta \) usage as well as \( \beta \) CDR3 length in comparison with \( \beta \)-chains expressed by bystander and normal T cell populations (16, 17). The technique of spectratyping has been developed to identify \( \beta \)-chain transcripts that exhibit these characteristics and has been used successfully to investigate the diversity of TCRs in sites of acute graft vs host disease and tumors in humans (16, 18, 19). The method involves \( \beta \) gene-specific PCR amplification, followed by electrophoresis of the products to identify overrepresented \( \beta \) genes and the lengths of products that are dependent on CDR3 lengths. In a number of cases, reamplification with \( \beta \)-specific primers restricts the products sufficiently to obtain CDR3 sequences directly from PCR products (16). Accordingly, spectratyping provides a rapid scan of all \( \beta \)-chain transcripts grouped according to the utilized \( \beta \) 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 cloned 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 \( \beta \) subfamily members that were represented prominently, but the removal of \( \beta \) CTLs by Ab-mediated depletion resulted in not only the use of alternative \( \beta \) 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 D\(^\alpha\)-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 was 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 \( \mu \)g/recipient) until rejection. A comparable regimen was used for depletion of V\( \beta \)8 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 V\( \beta \)8 \( \mathrm{T} \) 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 purifying columns (Applied Biosystems, Perkin-Elmer, Foster City, CA). Nested \( \beta \)-chain primers (Table I) were designed to amplify the noncoding C DNA 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. C\( \beta \)Seq corresponds to the first 20 bp of the 5’ end of the C\( \beta \) region. The C\( \beta \)Spectra primer is identical in sequence to C\( \beta \)Seq, but is labeled with 6-carboxyfluorescein (6-FAM) (Applied Biosystems) at the 5’ end. \( \beta \)-specific primers are homologous to specific regions of the \( \beta \) segment that distinguish different genes and subfamilies (24) and are described in Table I.
Vβ amplification and sequencing
cDNA was reverse transcribed from the CβNest I primer using the
Amplitrex 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
were added with sufficient sterile water to achieve a final volume of
9 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. 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 30 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. PCR products were gel purified (25) before
sequencing using the Prism Genescan version 2.0.1 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 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-

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

erved in normal T cell populations. However, alloantigen-specific
T cells should also be present and that population would be ex-
pected to exhibit a relatively reduced level of diversity of Vβ us-
age 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. If this were the case, spectra-
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 spectratyping 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
 manuscipt submitted). Spectratyping was performed on CTLs in-
filtrating 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 β 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 dif-
terent, 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 ad-

djacent 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 com-
plexity 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.
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$\beta$</th>
<th>CDR3</th>
<th>Sequence</th>
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<td></td>
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<td>Second-Set Allografts</td>
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<tr>
<td>2</td>
<td>A</td>
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<td>V$\beta$8.1-D-J$\beta$2.5</td>
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<td></td>
<td>J</td>
<td>$\beta$8.1-D-J$\beta$2.1</td>
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<th>CDR3</th>
<th>Sequence</th>
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<td></td>
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<td>I</td>
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</tr>
<tr>
<td>5</td>
<td>J</td>
<td>$\beta$8.1-D-J$\beta$1.1</td>
<td>V$\beta$8.1-D-J$\beta$2.5</td>
<td></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 Vβ8 subfamily PCR products, despite sequencing attempts on multiple non-Vβ8 products. The ability to obtain single-copy sequences directly from Vβ8 products suggested that 1) the number of different transcripts derived from these allografts was restricted despite apparent CDR3 length diversity within the Vβ8 PCR products, and 2) there was selective expansion of CTL-expressing Vβ8 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 Jβ segments bore a negative charge, which is greater than the 58% usage expected from unbiased Jβ usage (28). Eighty percent of all sequences contained two or more negative charges, which is distinctly different from characteristics of CDR3s specific for a Db 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 deoxyuridyl 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 Vβ8 subfamily usage with CDR3 length restriction, we assessed the effects of F23.1 Ab-mediated depletion of Vβ8+ 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 III. Vβ8 depletion by F23.1 Ab treatment has no effect on survival times of second-set male allografts

<table>
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<tr>
<th>Treatment</th>
<th>Number of Mice</th>
<th>Survival Times (days)*</th>
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<tr>
<td>F23.1</td>
<td>10</td>
<td>6.7,7.8,8,9,9,9,11</td>
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<tr>
<td>None</td>
<td>10</td>
<td>7.7,8.8,9,10,11,&gt;11</td>
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</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>
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<th>Mouse Peak</th>
<th>Vβ</th>
<th>CDR3</th>
<th>Sequence</th>
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<tr>
<td>7</td>
<td>2</td>
<td>9</td>
<td>C S A D P D S S Q D T L Y F</td>
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<tr>
<td>8</td>
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<tr>
<td>9</td>
<td>3</td>
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<tr>
<td>8 T</td>
<td>5</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>7 R</td>
<td>7</td>
<td>12</td>
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<td>9 V</td>
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<tr>
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</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-Vβ8 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 CASGDNASETLFY, 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-chain of the CTL-30 clone that was used to identify an HY peptide presented by Dβ1+ cells, which links this characteristic to in vitro derived, 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 pair α- and β-chain expression in individual CTLs in vivo, 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-Vβ8 Ab. This approach has been successful at retarding the rejection of allografts expressing H2L8 that has been shown to be preferentially recognized by Vβ8+ CTLs in vitro (29).

Anti-Vβ8 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.

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 strong 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 CASGDNASETLFY (14) and CASSDLVEYVFF (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...
5 due to a single codon shift in the site of recombination, since all three germline \textit{V}\textsubscript{b} 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 D\textsuperscript{b}-restricted CTLs, since two previously reported panels of influenza nucleoprotein-specific CTLs expressed \beta CDR3\textsubscript{s} with limited representation of any charged residues (8, 9).

The charge distribution in \beta CDR3\textsubscript{s} could be altered by pre-treatment of female recipients with anti-V\textit{\beta}8 Ab. This treatment reduced the frequency of V\textit{\beta}8-specific products; those that remained still exhibited the charge distribution that was characteristic of \beta CDR3\textsubscript{s} from untreated recipients. However, there was observed to be a considerable increase in \beta CDR3\textsubscript{s} 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 V$\beta$7 and the CASSGNTLYF CDR3 sequence. Therefore, V$\beta$8 depletion results in not only the usage of other V$\beta$ genes, but also alters the net charges of the expressed \beta CDR3\textsubscript{s}. It should be stressed that this alteration is not complete, in that \beta CDR3\textsubscript{s} with negatively charged residues in positions 4 to 6 can still be found with non-V\textit{\beta}8 genes, such as V$\beta$2, even after complete V$\beta$8 depletion.

The basis for the shift in net charge of \beta CDR3\textsubscript{s} is not clear at present, but may be related to the peptide(s) recognized by HY-specific CTLs. An HY peptide presented by D\textsuperscript{b} was predicted by identified mimotopes (13) and, more recently, has been identified and sequenced (6). This peptide, WMHHNDLII, was found in positions that allow binding of specific TCRs to the WMHHNDLII peptide. However, CDR3\textsubscript{s} joined to V\textit{\beta}8-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 \beta CDR3\textsubscript{s} 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 WMHHNDLII peptide. However, CDR3\textsubscript{s} joined to V$\beta$8 genes exhibit a strong net negative charge that would not appear compatible with such binding to the carboxy end of this peptide. The recent crystal structures of mouse and human TCRs (31, 32) suggest that \beta CDR3\textsubscript{s} are not in positions that allow binding of \beta CDR3\textsubscript{s} to the His residues of this peptide. It would appear that either the CDR3\textsubscript{s} of HY-specific V\textit{\beta}8 chains are not required for binding, or another peptide may be recognized by V\textit{\beta}8 CTLs. The former possibility appears unlikely given the marked, net negative charge and concentration of negative charges at positions 5–6 of these \beta CDR3\textsubscript{s} that are not observed in other D\textsuperscript{b}-restricted TCR (8, 9). Support for the latter possibility comes from the observation that CTLs expressing the published V\textit{\beta}8\textsuperscript{5} HY-specific TCR (14) do not recognize the WMHHNDLII peptide (E. Simpson, personal observation). If this were true, it would imply that anti-V$\beta$8 depletion resulted not only in a change in V$\beta$ usage, but also shifted the response to a second male-specific peptide that is recognized by CTLs that preferentially use the V$\beta$7 gene and express \beta CDR3\textsubscript{s} with characteristics, i.e., positively charged residues, favoring binding to that second peptide. Experiments are in progress to determine whether 1) HY-specific CTL clones expressing V$\beta$8 and V$\beta$7 actually recognize different peptides that can be eluted from D\textsuperscript{b} molecules and be separated chromatographically, and 2) a change in recognition of these peptides occurs following V$\beta$8 depletion in vivo. The results of these experiments will hopefully increase our understanding of the mechanisms underlying preferential T cell responses to immunodominant Ags.

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


