T Cell Receptor Genes in a Series of Class I Major Histocompatibility Complex-restricted Cytotoxic T Lymphocyte Clones Specific for a Plasmodium berghei Nonapeptide: Implications for T Cell Allelic Exclusion and Antigen-specific Repertoire

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Summary

We report here the first extensive study of a T cell repertoire for a class I major histocompatibility complex (MHC)-restricted cytotoxic T lymphocyte (CTL) response. We have found that the T cell receptors (TCRs) carried by 28 H-2Ka-restricted CTL clones specific for a single Plasmodium berghei circumsporozoite nonapeptide are highly diverse in terms of Vα Jα, and Jβ segments and aminoacid composition of the junctional regions. However, despite this extensive diversity, a high proportion of the TCRs contain the same Vβ segment. These results are in contrast to most previously reported T cell responses towards class II MHC-peptide complexes, where the TCR repertoires appeared to be much more limited. In our study, the finding of a dominant Vβ in the midst of otherwise highly diverse TCRs suggests the importance of the VB segment in shaping the T cell repertoire specific for a given MHC-peptide complex. As an additional finding, we observed that nearly all clones have rearranged both TCR α loci. Moreover, as many as one-third of the CTL clones that we analyzed apparently display two productive α rearrangements. This argues against a regulated model of sequential recombination at the α locus and consequently raises the question of whether allelic exclusion of the TCR α chain is achieved at all.

The mouse TCR α/β is a disulphide-linked heterodimeric integral membrane glycoprotein. Each chain of ~40-45 kD contains a C and a V extracellular domain (1). The diversity of each α and β V domain results from the somatic recombination of ~100 Vα with ~50 joining α (Jα) gene segments and of ~20 Vβ with two diversity β (Dβ) and 12 joining β (Jβ) gene segments, respectively. Imprecise joining and addition of template-independent N-nucleotides further contribute to this diversity (2). The TCR is closely related to Ig by similar domain organization, overall sequence homology, and conservation of key residues. Along this line, Chothia et al. (3) have proposed an outline of the TCR tertiary structure, based on the known three-dimensional structure of Igs.

Whereas B cells recognize epitopes on native antigenic proteins, T cells can only recognize antigens in the context of cell surface syngeneic MHC molecules (4). The antigens recognized by T cells can be mimicked by synthetic peptides (5). The crystallographic structure of two class I MHC molecules has revealed a groove in the external domain, where the antigenic peptide could lie (6-8). There is now evidence that the antigens are naturally processed into short peptides that are loaded onto MHC molecules and exported at the cell surface (9-11).

The T cell specificity for an MHC-peptide complex is determined exclusively by the TCR (12). Accordingly, one question has received much attention. What is the diversity of the TCRs carried by T cells of a given specificity?

The determination of the primary structure of a number of TCRs carried by T cell clones or hybridomas of a given specificity, mostly MHC class II restricted, has been performed for a variety of protein antigens, either of eucaryotic origin, such as pigeon cytochrome c (pcc) (13-19), beef insulin (Bi)
were apparently productive.

response, we found that although the TCRs were highly di-
verse in terms of V~ and Jc~ segments and amino acid
composition of the junctional regions, the response was para-
adoxically dominated by a VB segment. We also observed that
Vol and 20 V~/oligonucleotides in conjunction with a Ca
nearly all clones had rearranged both TCR ot loci and that
screened each T cell clone by PCR with a collection of 19
in vitro with the 12-mer PbCS 249-260 and their lymph node clones stimulated in vitro with the same peptide (CTL E, F, and M; total of seven clones); (d) (BALB/c × C57Bl/6)F, mice immunized intrave-
ously with a lipid derivative of the 9-mer PbCS 252-260 and their spleen cells stimulated in vitro with the 12-mer PbCS 249-260 (CTL H and L; total of four clones). (e) BALB/c mice immunized intra-
veously with the above lipopeptide and their spleen cells stimulated in vitro with the 12-mer PbCS 249-260 (CTL K; one clone). (f) (BALB/c × C57Bl/6)F, mice immunized with the 8-mer PbCS 253-260 and their lymph node clones stimulated in vitro with the same peptide (CTL I and J; total of five clones). The details of in vivo priming and in vitro stimulation and cloning are reported elsewhere (35, and Romero et al., manu-
script in preparation). Cloned cells were harvested 5-7 d after
the last restimulation for cell surface staining or for RNA extraction.

Cell Surface Labeling with VB-specific mAbs. CTL clones were cultured with several anti-VB mAbs: F23.1 (anti-Vb8.1, 8.2, 8.3), F23.2 (anti-Vb8.2) (39), KJ16 (anti-Vb8.1, 8.2) (40), 44-22-1 (anti-
Vb6) (41), Mm VB-TCR-6B (anti-Vb13) (PharMingen, San Diego,
CA), 14-2 (anti-Vb14) (42) and TR310 (anti-Vb7) (43). Briefly,
cells were stained with saturating amounts of the anti-VB mAb
followed by an appropriate FITC-labeled anti-Ig second reagent
or Avidin-PE. Samples were passed on a FACS II flow cytometer
(Becton Dickinson & Co., Sunnyvale, CA).

RNA Extraction, DNA Synthesis, and PCR. Total RNA was
taken from 10^6 cells by disruption of the cells in guanidinium
thiocyanate followed by ultracentrifugation through a cesium
chlo-
cide cushion. Pelleted RNA was thereafter extracted once with
phenol-chloroform, ethanol precipitated, and suspended in 50 μl
water. Single-stranded cDNA synthesis was carried out on 5 μg
total RNA with oligo(dT)15 and AMV reverse transcriptase
(Boehringer Mannheim Biochemicals, Indianapolis, IN) according
to manufacturer's instructions. After an ethanol precipitation,
the cDNA was suspended in 100 μl water. PCR was carried out in
100 μl on 1/100 of the cDNA with 1.5 U of Taq polymerase (Cetus
Corp., Emeryville, CA) according to manufacturer's instructions.
The primers are listed in Table 1. 30 cycles, each of 95°C for 1
min, 55°C for 1 min, and 72°C for 1 min, were completed in a
thermostater (Cetus Corp.).

Direct Sequencing of Double-stranded Linear DNA. The PCR
products were ethanol precipitated and separated by electropho-
resis on a 2% agarose gel. The band of interest was cut out, sub-
mited to electroelution, and the eluted DNA ethanol precipitated.
This double-stranded linear DNA was directly sequenced with the
Sequenase version 2.0 kit (United States Biochemicals, Cleveland,
OH) and 3′dATP as described (38). Briefly, 0.4 pmol of template
was boiled for 10 min in 10 μl with a 20-fold molar excess of se-
quencing primer. The sample was rapidly transferred to a dry-ice
ethanol bath. Labeling mix (5.5 μl) was added to the frozen pellet
and the tube allowed to warm. Once the ice melted, the solution
was incubated for ~30 s at room temperature and then four 3.5-μl
samples were transferred to the borders of wells containing 2.5
μl of the respective ddNTPs mixtures. The 96-well plate was then
spun down and incubated at 37°C for 2 min. The reaction was
stopped by 5 μl of stop solution. The sequence products were sepa-
rated on an 8 M urea, 6% acrylamide gel.

Probabilities. Let us consider that a rearrangement has a proba-
bility $p_1$ to be productive: $p_1$ depends on both the frequency of germline V, (D), and J pseudogenes and the frequency of unproductive V(D)J recombinations. Let us also consider a probability $p_2$ for a productive rearrangement to shut off the recombination of the other locus: $p_2$ depends on hypothetical post translational requirements, e.g., level of expression, heterodimer formation, cell surface expression, positive selection, etc. In any case, if the two $\alpha$ loci rearrange sequentially and if the recombination is regulated, the proportion of cells with one productive plus one unrearranged ($\alpha~\gamma\alpha$) or two productive ($\alpha~\alpha$) rearrangements would be: $1/(2 - p_1p_2)$, $(1 - p_2)p_1/(2 - p_2p_2)$, and $(1 - p_1)/(2 - p_1p_2)$, respectively. Thus, since $F_1$ must be a value between 0 and 1/3, and $\alpha_3$ between 0 and 1, these proportions can be estimated to be between 1/2 and 3/5, 1/6 and 0, and 1/3 and 2/5, respectively. Our observed values for any of the three categories differ significantly from these expected values. Even if we cannot rigorously discriminate between productive and unproductive rearrangements because we do not have the full-length sequences, it is established that only 4 of 28 CTL clones, at most, have still one unrearranged $\alpha$ locus, instead of 14-17 as expected. Consequently, a regulated model of sequential recombination is unlikely to hold at the TCR $\alpha$ loci.

**Results**

A collection of 47 CTL clones, H-2K<sup>d</sup> restricted and specific for the PbCS nonapeptide 252-260, was derived from 13 mice and is described elsewhere (35, 36; Romero et al., manuscript in preparation). The combination of different mice and V$\beta$ cell surface expression, as determined by fluorescence staining (see Materials and Methods), indicated that at least 23 clones were independent. All CTL clones were then tested by cDNA-PCR with a sense oligonucleotide specific for the V$\beta$ determined by FACS analysis and an antisense CB oligonucleotide (Table 1), followed by direct sequencing of the

### Table 1. Oligonucleotides

| V$\beta^*$ | Sequence (5' to 3') | V$\alpha^*$ | Sequence (5' to 3') |
|-----------|---------------------|-------------|---------------------|
| V$\beta_1$ | CCCAGTCGTTTTATACCTGAATGC | V$\alpha_1$ | GCACTGATGTCATCTTCTC |
| V$\beta_2$ | TCACGATACGGAGCTGAGGC | V$\alpha_2$ | AAAGGAGAAAAAGCTTCC |
| V$\beta_3$ | CTTGTCAGGAAATTCATGCC | V$\alpha_3$ | AAGTACTATTCCGGAGACC |
| V$\beta_4$ | GCCCTCAAGGCTCCACCC | V$\alpha_4$ | CAGTATCCGGAGAGTC |
| V$\beta_5.1$ | GTCACAAGTTTATGATGCTACAC | V$\alpha_5$ | CAAAGGAGACAAGACTTCT |
| V$\beta_5.2$ | AAGGAGAGAGAGAATGAGGC | V$\alpha_6$ | ATGGCTTTCTCGGTATTGCC |
| V$\beta_6$ | CTCTACATGGATGACCTGCC | V$\alpha_7$ | TCTGTATGCTCAGAAGTC |
| V$\beta_7$ | TACAGGGTCTCAGGAAGAG | V$\alpha_8$ | CAAAGAAGGGAGCACACC |
| V$\beta_8.1$ | CATTTCGAGTTGCTTCCC | V$\alpha_9$ | TAGTGACGTGTTGATGTC |
| V$\beta_8.2$ | CCTCATCTGGAGTTGGCTACCC | V$\alpha_{10}$ | AAGGAGCAGCAGCTTGCAC |
| V$\beta_8.3$ | ACAGGAAAGAGACTCTTCTCTGCTG | V$\alpha_{11}$ | CCGGACATCGAGGATGCC |
| V$\beta_9$ | TCTCTCTCATGGCTGCGGACCG | V$\alpha_{12}$ | TCTGTATGCTCAGAAGTC |
| V$\beta_{10}$ | ATCAAGTCTGTAGAGGCCGAGG | V$\alpha_{13}$ | ND |
| V$\beta_{11}$ | GCACGTCACCTCGGAGATCCAGG | V$\alpha_{13.1}$ | ACCTGGAGAAGATCTCAG |
| V$\beta_{12}$ | GAAATGCTGGTGGGCTTTACAGATC | V$\alpha_{34S-281}$ | TCCTGGTGGACAAAGAAC |
| V$\beta_{13}$ | AGGGCTAAGAGATCTAAGTCAC | V$\alpha_{10}$ | TGGTTTGAGGACAGTGGG |
| V$\beta_{14}$ | ACGGACAAATCTCCTTGAC | V$\alpha_{BW}$ | CATTGCCTAAATGGAAGAG |
| V$\beta_{15}$ | CCCATCAGTCATCCAACTTATTCC | V$\alpha_{MA}$ | CAAATGAGGAGAGAGAAG |
| V$\beta_{16}$ | CACTCTGAAATCTCAGCCAC | V$\alpha_{MB}$ | GGAAATGCAACAGTGGGTC |
| V$\beta_{18}$ | CAGCCGCCCAACCTAACAATTTGCT | V$\alpha_{ST}$ | GACATGACGCTTCTGAGGCTTGC |
| C$\beta$ | Sequence (5' to 3') | C$\alpha$ | Sequence (5' to 3') |
| C$\beta_a$ | CCAGAAGGTAGCAGAGACC | C$\alpha_a$ | TGGCGTGGTGCTCCTTGAAG |
| C$\beta_b$ | CTTGGTGTGGAGTCACATTTCTC | C$\alpha_b$ | ACACAGCAGGTTTCTGGGTC |

* $V\beta$ primers are sense oligonucleotides designed according to each $V\beta$ gene segment. The $V\beta$ gene segment nomenclature follows that of references 51, 67, 68, and 69.

* $V\alpha$ primers are consensus sense oligonucleotides designed according to the known members of each $V\alpha$ subfamily. The $V\alpha$ gene subfamily nomenclature follows that of references 2, 45, 75, and 76. Nucleotide sequence of the $V\alpha_{13}$ subfamily (2) was not available. The $V\alpha_{15}$ gene segment sequence and specific primer were kindly provided by P. Marche (Institut Pasteur, Paris, France).

* The C$\beta$ primers are consensus antisense oligonucleotides for the C$\beta_1$ and C$\beta_2$ genes (71, 72). C$\beta_b$ is located 3' to C$\beta_a$.

* The C$\alpha$ primers are antisense oligonucleotides designed according to the single C$\alpha$ gene (77, 83). C$\alpha_b$ is located 3' to C$\alpha_a$. 

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### A

| CTL clone | vB |
|-----------|----|
| B28       | vB3.3 |
| B18       | vB7  |
| M1        | vB1  |
| RA1.0.3.3 | vB3  |
| C1        | vB2  |
| F12       | vB4  |
| CI1       | vB1  |
| F1        | vB1  |
| J1        | vB1  |
| QA1.1.2.2 | vB2  |
| F12        | vB1  |
| CI1       | vB1  |
| F1        | vB1  |
| J1        | vB1  |
| QA1.1.2.2 | vB2  |
| F12        | vB1  |
| CI1       | vB1  |
| F1        | vB1  |
| J1        | vB1  |
| QA1.1.2.2 | vB2  |
| F12        | vB1  |
| CI1       | vB1  |
| F1        | vB1  |
| J1        | vB1  |

### B

| CTL clone | vB |
|-----------|----|
| B28       | vB3.3 |
| B18       | vB7  |
| M1        | vB1  |
| RA1.0.3.3 | vB3  |
| C1        | vB2  |
| F12       | vB4  |
| CI1       | vB1  |
| F1        | vB1  |
| J1        | vB1  |
| QA1.1.2.2 | vB2  |
| F12        | vB1  |
| CI1       | vB1  |
| F1        | vB1  |
| J1        | vB1  |
| QA1.1.2.2 | vB2  |
| F12        | vB1  |
| CI1       | vB1  |
| F1        | vB1  |
| J1        | vB1  |
| QA1.1.2.2 | vB2  |
| F12        | vB1  |
| CI1       | vB1  |
| F1        | vB1  |
| J1        | vB1  |

### C

**Figure 1.** TCR α and β DNA junctional nucleotide sequences. (A) TCR β DNA junctional sequences. Out-of-frame sequences are indicated by an asterisk. Nomenclature for Vβ gene segments follows that of references 67-69 and 51. Sequences are in references 67 (Vβ1, Vβ6, Vβ7), 70 (Vβ10, Vβ12, Vβ13), 68 (Vβ14, Vβ16), and 51 (Vβ5.2, Vβ8.1, Vβ8.2). The β sequences are from references 71 and 72. The Vβ and Jβ gene segment sequences are identical to the published ones. However, two Jβ genes differ from the original reports: there is a base insertion (underlined) in the Jβ15 segment (...AACAGGCCTCCG...) and a base substitution (underlined) in the Jβ14 segment (...CTGTCGTGTCCG...). Each of these changes occurred in all respective CTL clones. The Dβ7 segments are underlined (73, 74). The Cβ1 and Cβ2 genes could not be discriminated on a one-base basis and probably follow the Jβ cluster to which the Vβ is rearranged (71, 72). (B) TCR α cDNA junctional sequences. Out-of-frame sequences are indicated by an asterisk. Nomenclature for the Vα gene subfamilies follows that of references 2, 45, and 76. The Vα subfamily is separated from the Vγ gene segment by a period. The Vα gene segments are named according to the original cell in which they were isolated, from references 77 (3.pHDSS58), 45 (5.TA72, 10.TA57, 6.TA1, 2.TA19), 78 (5.MDA, 4.MDI3, 1.Em), 14 (4.5), 46 (8.F2.2, 8.F3.3, 8.F4.3, 8.F5.3), 79 (7.B24Exp), 33 (3.8B19), 21 (BMB24H11), 26 (2.81), 80 (BMA.P14), 81 (4.6R), 24 (4.PJ.R25), 75 (7.2), 34 (3.A5R), 17 (10.1F8), 82 (2.2), 60 (5.MS202), 23 (11.3), and this report (1.B83, 5.TJ3, BMA.H3, 2.F1, A.1125, 4.F2101, BMA.M2, F3103). The Jα gene segments are from references 43 (TJ10), 77 (p.HDSS58), 78 (L2B, MD13, B4, C5), 45 (TA27, TA80, TA19, TA28, TA37, TA65, TA39, TA57, TA61, TA1, TA31), 84 (BD31), 14 (4.4), 32 (11.2), 53 (20K), 75 (3.DT), 59 (A10), 85 (14T), 68 (11-12-27), and this report (B28, B732, 17, H2, K1, C7). The Vγ and Jα gene segment sequences are identical to the published ones. However, the JαMD13 in clone J4 differs by a silent base substitution (underlined) from the original JαMD13 (...TTCGGCCG...). These sequences are available from EMBL/GenBank/DDBJ under accession numbers X60857 to X60921.
double-stranded PCR product. This procedure allowed a rapid
determination of putative sister clones, i.e., CTL clones iso-
lated from the same mouse and displaying an identical TCR
β gene sequence. Thus, 28 independent CTL clones could
be identified unambiguously (Fig. 1 A). All studies described
below were carried out on these 28 clones.

Few CTL Clones Express Two Distinct VDJC β Transcripts.
For each clone, the nucleotidic sequence of the amplified
cDNA revealed an open reading frame encoding TCR β chain
key residues (Fig. 1 A and Fig. 2). To further document these
findings, we performed cDNA PCR on each CTL clone with
a collection of 20 oligonucleotides specific for each of the
known functional Vβ gene segments of the Vβb haplotype
(Table 1). All these primers were shown to amplify the respec-
tive Vβ gene segment under the experimental conditions used.
6 of 28 CTL clones were found to express two different VDJC
β transcripts. However, only one transcript was productive
in every case (Fig. 1 A and Table 2). Since most if not all
Vβ gene segments are known (44), the combination of cell
surface stainings and nucleotide sequences indicated that the
transcripts encoding the functional β chains were unambigu-
ously determined (Fig. 2).

Nearly All CTL Clones Express Two Distinct VJC α Trans-
cripts. We analyzed the VJC α transcripts by cDNA-PCR
followed by direct sequencing. Each CTL clone was tested
separately with 19 consensus oligonucleotides designed ac-
cording to the known members of 19 V α subfamilies in con-
junction with a C α oligonucleotide (Table 1). In contrast
to the β transcripts, 23 of 28 CTL clones were found to ex-
press two different VJC α transcripts (Fig. 1 B). One of the
two CTL clones in which only one VJC α transcript was
detected, C1, contained an out-of-frame transcript, and thus
presumably expressed a second undetected in-frame transcript.
Another CTL clone, J5, was found to express an out-of-frame
VDJC α transcript (Casanova et al., manuscript in prepara-
tion). The remaining three clones, C11, J2, and L4, could
possibly retain one unrearranged α locus. However, given
the large estimated number of Vα gene segments and pos-

| CTL clone | Vβ | FW | CDR3 | FW | Jβ |
|-----------|----|----|------|----|----|
| B28       | 7  | CAS| S S R Y E Q | YFG | 2.7 |
| M1        | 14 | CAW| G T G G F A E Q | FFG | 2.1 |
| RA10.3.3  | 14 | CAW| S K G A R G Q N T L | YFG | 2.4 |
| B83       | 6  | CAS| T P T G T N N Q A P | LFG | 1.5 |
| C11       | 6  | CAS| I P T A N T G Q L | YFG | 2.2 |
| F12       | 6  | CAS| I G T G G G T Q G Q L | YFG | 2.2 |
| C1        | 8.1| CAS| S V T G S N T E V | FFG | 1.1 |
| H3        | 8.1| CAS| S D S Q G T E V | FFG | 1.1 |
| F1        | 8.1| CAS| S D E G V G E N T L | YFG | 2.4 |
| QA11.3.2  | 8.1| CAS| R P G Q P Y E Q | YFG | 2.7 |
| I7        | 8.2| CAS| G D G N Q A P | LFG | 1.5 |
| F8        | 8.2| CAS| G T G G G A N T G Q L | YFG | 2.2 |
| E22       | 13 | CAS| S P T G K S N T E V | FFG | 1.1 |
| QB7.3.2   | 13 | CAS| S P P Q V A N T E V | FFG | 1.1 |
| H2        | 13 | CAS| S P T G R N T E V | FFG | 1.1 |
| K1        | 13 | CAS| R R Q G G T E V | FFG | 1.1 |
| F15       | 13 | CAS| S P P Q G N Q D T Q | YFG | 2.5 |
| FF2.10.1  | 13 | CAS| S F R G G Q D T Q | YFG | 2.5 |
| H1        | 13 | CAS| S S A R G D T Q | YFG | 2.5 |
| J3        | 13 | CAS| R D R G R T N E R L | FFG | 1.4 |
| J5        | 13 | CAS| S S P Q G S N E R L | FFG | 1.4 |
| M2        | 13 | CAS| S L G Q G A F N Y A E Q | FFG | 2.1 |
| J2        | 13 | CAS| S F R D R G N Y A E Q | FFG | 2.1 |
| L4        | 13 | CAS| S R L G A S Y E Q | YFG | 2.7 |
| PF5.1.1   | 13 | CAS| S F Q Y E Q | YFG | 2.7 |
| J4        | 13 | CAS| S R R D R D Q A P | LFG | 1.5 |
| C7        | 13 | CAS| S P G Q G L T G Q L | YFG | 2.2 |
| RF3.10.3  | 13 | CAS| S S A S A E T L | YFG | 2.3 |

Figure 2. TCR β chain junc-
tional amino acid sequences. 28
CTL clones are listed on the ver-
tical axis. For each clone, the TCR
β transcript with both an open
reading frame and the triplets
coding for key residues at the VDJ
junction (3) was considered to en-
code the functional TCR β chain.
For all clones (except B28, not
stained by the anti-Vβ7 mAb),
the FACS stainings with anti-Vβ
mAbs were in strict agreement
with the β transcript assignments. The
deduced amino acid sequences (in
single-letter amino acid code) of the
junctional, hypervariable and puta-
tively CDR3-like regions, ac-
cording to Chothia et al. (3), are
represented. The presumed Ig-like
loop, designated CDR3 for conve-
nience, is putatively supported by
two framework branches (FW).
Table 2. Status of the TCR α and β V(D)JC Transcripts

| Status* | α  | β  |
|---------|----|----|
| + / -  | 17 (61%) | 6 (22%) |
| + / +  | 8 (29%) | 0 |
| + / 0  | 3 (10%) | 22 (78%) |

* The symbols + and − indicate transcripts that exhibit or not, respectively, an open reading frame at the V(D)JC junction, and the symbol 0 indicates the absence of transcript detected by cDNA PCR.
† Clones M2 (+/0) and J5 (+/0) are included in this category because they express either a nonfunctional, although in-frame, α transcript, due to the absence of the triplet coding for the key Cys residue at position 90, or a δ transcript, respectively. Clone C1 (0/0) is also included because it bears an α/β TCR on the cell surface and must therefore express a productive α transcript in addition to the unproductive one detected (see Fig. 3 A).
‡ The three clones C11, J2, and L4 might express an additional α transcript that would not hybridize to the PCR primers (see Fig. 3 B).

The functional TCR α and β chain repertoires are highly diverse. Six Vβ gene segments from five different subfamilies are represented (Fig. 2). Vβ13 is predominant, found in 16 CTL clones (57%). The frequency of Vβ13 is lower among CTL from peptide-immunized mice (1/5). Among CTL clones from peptide-immunized animals, the frequency of Vβ13 usage is 74% (15/23). The Vβ13 frequency among CD8+ lymphocytes in these strains of mice is between 3% and 5% (data not shown). Furthermore, the Vβ13 predominance is unlikely to be the result of an increased representation of Vβ13 among H-2Kb-restricted T cells, since only one Vβ13 usage was found in the analysis of 25 independent H-2Kb-restricted CTL clones specific for other peptides (data not shown).

The TCR β Chain Primary Structures Are Otherwise Highly Diverse. All 28 TCR β chains differed from each other, and apart from the Vβ13 usage, no other predominant structural features were identified for the β chain repertoire. Indeed, 10 Jβ segments, out of 12 possible genomic segments, are used among 28 CTL clones. Even among the 16 Vβ13 CTL clones, eight different Jβ segments are used. Likewise, no preferential Jß segment usage was observed for the other Vβ segments. Furthermore, the length of the CDR3 loop, defined according to Chothia et al. (3), varies from 6 to 12 amino acids, without striking dominant intermediate values. The CDR3 lengths are also highly variable among CTL clones that share a given Vβ segment and even among those that share a given Vβ-Jß pair. When all loops of a given length were compared for the amino acid composition at a given position, no conservation could be found. Even when positions were assigned with respect to the Cys 92 residue (3), loops with different lengths did not show any obvious amino acid conservation. Moreover, even loops of a given length and supported by a given Vβ or Jß framework failed to show amino acid conservation in the non-Vβ- or non-Jß-encoded regions, respectively.

The Functional TCR α Chains Are Also Highly Diverse. The 16 known functional TCR α chains were found to differ from each other (Fig. 3). No Vα subfamily predominance such as the Vβ 13 among Vβs was observed. Overall, 8 different Vα subfamilies, 13 different Vα gene segments, and 13 different Jα segments were found among the 16 CTL clones.

Table 3. Gene Segments Used by Unambiguous Functional TCR Heterodimers

| CTL clone* | Vβ1 | Jβ | Vα | Jα |
|------------|-----|----|----|----|
| B28        | 7   | 2.7 | 8.F3.3 | LB2 |
| RA10.3.3   | 14  | 2.1 | 8.F3.3 | TA27 |
| B83        | 6   | 1.5 | 8.F3.4 | LB2 |
| F12        | 6   | 2.2 | 8.F3.3 | TA80 |
| F1         | 8.1 | 2.4 | 7.62BExp | 14.4 |
| QA11.3.2   | 8.1 | 2.7 | 4.A1132 | 14.4 |
| I7         | 8.2 | 1.5 | 5.MDA | 17 |
| F8         | 8.2 | 2.2 | 6.TA1 | TA65 |
| F15        | 13  | 2.5 | 3.810 | TA57 |
| H1         | 13  | 2.5 | 4.3 | TA61 |
| J3         | 13  | 1.4 | 5T.J3 | TA27 |
| J5         | 13  | 1.4 | 4.3 | TA1 |
| M2         | 13  | 2.1 | BMA.M2 | TA39 |
| J4         | 13  | 1.5 | BMA.P14 | MD13 |
| C7         | 13  | 2.2 | 3.pHDS58 | TT11 |
| RF3.10.3   | 13  | 2.3 | 4.F3103 | TT11 |

* For 16 CTL clones, the functional α chain, engaged in heterodimeric formation with the β chain and specific of the H-2Kb-PB95-260 combination, was unambiguously determined. Indeed, 14 clones express a second, out-of-frame, α transcript. In addition, clone J5 expresses a δ transcript, and one of the two in-frame α transcripts from clone M2 has deleted the triplet coding for the key Cys residue at position 90.† Nomenclature and references for the Vβ, Jβ, Vα, and Jα gene segments are in the legend to Fig. 1.
Figure 3. TCR α chain junctional amino acid sequences. The 28 CTL clones are separated in three groups, as in Table 2. Group A (17 clones) gathers clones for which the functional α chain can be unambiguously assessed due to the presence of a second, out-of-frame, α transcript. It also includes clone J5, which expresses a β transcript, and clone M2, which expresses two in-frame α transcripts, one of them being, however, nonfunctional since it has deleted the triplet coding for the conserved Cys residue at position 90. Clone C1, for which we could not detect a productive α transcript, is also included. Group B (three clones) gathers clones for which only one α transcript was detected and found to be productive. Thus, we can not affirm that the corresponding α chain is necessarily functional. Group C (eight clones) gathers clones that express two α transcripts in frame at the VJ junction, both potentially encoding a functional α chain. For each group, the α chain deduced amino acid sequence (in the single-letter code) of the CDR3-equivalent loop, according to Chothia et al. (3), is reported with the Vα and Jα segments (for references see Fig. 1). The key Cys residue is at position 90 in the α chain.

The CDR3 lengths were found to be extremely variable, ranging from 4 to 11 amino acids and without any particular distribution. Moreover, no obvious amino acid conservation was found, even when loops of a given length and/or supported by a given Vα or Jα framework were compared. When all different possible α chains from the CTL clones for which the functional α chain was only putative were included in the analysis, the structural diversity was further increased. Altogether, the TCR α chain primary structures were found to be highly diverse.

Discussion

We have analyzed the TCRs from 28 independent CTL clones specific for the PbCS nonapeptide 252-260 presented by the H-2K2 restriction element. The sequences of the amplified TCR α and β cDNAs were determined. From this analysis, two major observations were made. First, nearly all CTL clones have rearranged both TCR α loci, and as many as one third of these clones apparently display two productive α rearrangements. Second, although all TCR α and β chains differ from each other and are highly diverse in terms
of both Vα, Jα, Jβ segments and amino acid composition of the junctional regions, where no conserved amino acid was found, there is a strong dominance of the Vβ13 segment.

**Rearrangements of the VDJ β Gene Segments.** All CTL clones express only one productive VDJC β transcript and a few (22%) express an additional unproductive one (Table 2). This study at the mRNA level by cDNA-PCR provides reasonably reliable information on genomic VDJ rearrangements. Similar proportions at the genomic level are described for the Ig H chain locus in B cells, where a regulated model of sequential recombination is believed to account for allelic exclusion (48-50). In this model, a complete VDJ rearrangement occurs first on one chromosome, and only if this is not productive can the other locus then rearrange. Such a model predicts that on the average 40-50% of peripheral cells should contain two distinct VDJ rearrangements (see Materials and Methods). The somewhat lower level (22%) in the present study could be explained in part by rearrangements involving a Vβ pseudogene of the Vβb haplotype (44, 51, 52), for which we did not design specific oligonucleotides. Since most if not all TCR Vβ gene segments are presumably described, rearrangements involving new Vβ segments are very unlikely (44). Surprisingly, two mouse T cell clones harboring two productive β rearrangements have recently been reported (53, 54). The relatively large series analyzed here suggests that the frequency of such cells is very low and that consequently they may have little biological significance. Altogether, the TCR β gene VDJ rearrangements appear to be consistent with a regulated model of sequential recombination that maintains a strict allelic exclusion for the TCR β chain.

**Rearrangements of the V/β Gene Segments.** In contrast to our findings for the β transcripts, nearly all CTL clones analyzed express two distinct Vβ VJC transcripts (Table 2). Few previous studies have analyzed the status of rearrangements at both α loci in T cell clones, mainly because of the size of the Jα locus, which extends over a stretch of 60 kb and thereby makes the analysis by Southern blot difficult and sometimes ambiguous (55-58). In one study, only 3 of 10 T cell clones were reported to be rearranged at both α loci (58). In contrast, Malissen and colleagues (55) reported that both α loci were rearranged in eight of nine T cell clones. Our cDNA-PCR approach with an extensive series of Vα primers followed by sequencing has allowed us to demonstrate unambiguously that at least 24 of 28 clones have rearranged both α loci. Thus, it is likely that both TCR α loci are generally rearranged in most peripheral α/β T cells.

At least 8 of the 28 CTL clones (29%) specific for the *Plasmodium berghei* CS nonapeptide appear to express two productive α transcripts. In the literature, three T cell clones have been described that clearly bear two productive α rearrangements (59-62), but in the absence of an extensive study to estimate the frequency of such cells, their biological relevance was unclear. Our study now indicates that the occurrence of T cells that express two productive α transcripts is probably rather frequent. Moreover, it raises the question of whether TCR α chain allelic exclusion (i.e., the presence of a single heterodimer at the cell surface) is actually achieved in such lymphocytes.

Altogether, these results on the TCR α gene VJ rearrangements strongly suggest that the recombination events at the TCR α loci differ considerably from those at the Ig and TCR β loci, and that a regulated model of sequential recombination may not hold for the TCR α genes (see Materials and Methods).

**Diversity of the TCR α and β Chains.** Several class II MHC-restricted helper T cell responses to well-defined peptides have been reported. For some antigens, the number of sequences is too low to give an idea of the actual repertoire. For those studies with a sufficient number of sequences, there is clearly the occurrence of not only a dominant Vβ segment but also an associated dominant Jβ segment, a common CDR3 length, and a conserved amino acid in the non-Vβ-, non-Jβ-encoded part of the CDR3 loop (Table 4). However, the diversity of the repertoire varies slightly from case to case and the response to HA appears to be the most diverse. In addition, a very striking feature is the fact that T cell clones bearing TCR β chains identical at the amino acid level could be isolated in all these studies from different individual mice at a high frequency. In contrast, apart from the Vβ13 dominance, we found no structural limitations in the CTL response to the *P. berghei* CS nonapeptide-K^d^ complex and no identical β chains, despite a larger number of clones analyzed.

A striking recurrence of particular combinations of Vα gene subfamily, Jα segment, and CDR3 length has been observed in most class II MHC-restricted T cell responses analyzed (Table 5). Most strikingly, T cell clones bearing identical α chains were isolated from different individual mice. This is again compelling evidence for a very strong selective pressure, given the potential diversity of the receptor chains. As an exception, the response to HA appears to be more diverse. However, the function of the proteins encoded by the α transcripts in the latter study is only putative, since data on the second TCR α locus are lacking. In the present study, in which we analyzed not only many but, most importantly, unambiguously assigned functional α chains, we found no dominant Vα usage, no limitations on the junctional structures, and no identical α chains.

For the 16 CTL clones in our study where the functional α/β pairing is unambiguous (Table 3), there is no obvious preferential Jα-Jβ pairing, nor Vα-Vβ pairing. Rather, there is a large diversity of pairing between chains encoded by different Vα and Vβ gene subfamilies or segments. Thus there are 11 or 15 different α/β pairs, considering V gene subfamilies or segments, respectively, out of 16 TCRs. Notably, the Vβ13 and Vβ8 subfamily-encoded chains appear to be mutually exclusive with those of Vα8. If the remaining 12 clones are included in the analysis, the diversity of pairing is further increased. In contrast, most other T cell responses analyzed to date display limited pairing diversity. Of these, the HA response appears to be the most diverse, but again, the α chains are only putative and so the pairing among functional chains could possibly be less diverse. In many studies, TCRs identical not only for one chain but for both the α and β chains were isolated from different individual mice (Table 5).

Each of the 28 CTL clones has a unique fine specificity pattern when tested for recognition of a series of Ala-
### Table 4. Comparison of the TCR β Chain Repertoires Reported for Various Antigens

| MHC-peptide complex | Strain | Total TCR β | Identical TCR β | Dominant Vβ (n) | Dominant Jβ (n) | Dominant length (n) | Conserved amino acid** |
|---------------------|--------|--------------|----------------|----------------|----------------|---------------------|------------------------|
| MHC class II        |        |              |                |                |                |                     |                        |
| pccs01.10/IEk        | B10.A, F1/A | 15   | 2,2,2,2,2  | Vβ3 (7) | Jβ1.2 (5) | 9 (5) | N         |
|                     |        |              |                |                |                |                     |                        |
| pccs01.10/IEk       | B10.S9R, F1/9R | 7    | 2       | Vβ1 (6) | Jβ1.2 (5) | 8 (5) | D         |
| SpWMb10-121/IEd     | DBA/2  | 6    | 3       | Vβ8.2 (6) | Jβ2.7 (5) | 10 (5) | WDW       |
| MBP59.9k/IA*        | PL/J, (P x S) F1 | 8    | 2,2,2   | Vβ8.2 (7) | Jβ2.7 (4) | 8 (4) | GLG       |
|                     | B10.PL | 6    | 4       | Vβ8.2 (5) | Jβ2.7 (5) | 9 (5) | AGG       |
| αC12-26/IEk         | A/J    | 11   | 3,2     | Vβ1 (8) | Jβ2.1 (7) | 10 (7) | E         |
| HA110-120/IEd       | BALB/c | 13   | 3       | Vβ8.3 (7) | Jβ1.3 (4) | 8 (4) | G         |
| MHC class I         |        |              |                |                |                |                     |                        |
| PbCS252-260/Kd      | BALB/c, (B x C)F1 | 28   | NF      | Vβ13 (16)  | NF            | NF       | NF         |

* For references, see Introduction. Only studies with at least six sequenced TCRs specific for a given MHC-peptide complex are included. Thus, studies on HEL-, Bi-, pcc (B10S[9R])- and LCMV-specific T cells are not reported. Studies on hapten-specific T cells are not mentioned because the processed form of the antigen is unknown.

1 Responses to a given MHC-peptide combination in different strains are considered separately, with the exceptions of chimeras (F1/A [(B10A x B10S[9R])F1 bone marrow in B10.A irradiated host] and F1/9R [(B10.A x B10.S[9R])F1 bone marrow in irradiated B10.S[9R]], as well as hybrids (P x S)F1 [(PL.J x SJL)F1] and (B x C)F1 (BALB/c x C57BL/6)F1).

2 Only sequenced TCR β chains are considered. The numbers indicate the total number of sequenced β chains for each particular specificity and strain. Probable sister clones, i.e., clones displaying the same TCR gene sequence and coming from the same animal, are excluded.

3 Only identical TCR β chains isolated from different individual mice are reported, irrespective of the Vβ or Jβ usage. Each number indicates how many chains share a given structure. For example 3,2 means that two distinct groups of identical chains were found, one with three members, and one with two members.

4 Any element, gene segment (Vβ, Jβ) or CDR3 length (3), is said to be dominant if it represents more than a third of the previous element(s), in the order of the Table. The nature of these elements in each case is given in the Vβ, Jβ, and length columns, respectively. n indicates the number of TCRs sharing this element among the ones carrying the previous one(s).

** Conserved amino acid indicates the non-Vβ, non-Jβ-encoded conserved residue (single-letter code) in the CDR3 loop among TCRs sharing the previous dominant Vβ-Jβ-length combination (only N in the pcc [B10.A] response is not absolutely conserved, present in four out of five TCR β chains).

### Table 5. Comparison of the TCR α Chain Repertoires Reported for Various Antigens

| MHC-peptide complex | Strain | Total TCR α | Identical TCR α | Identical TCRα/β | Dominant Va (n) | Dominant Jα (n) | Dominant length (n) |
|---------------------|--------|--------------|----------------|-----------------|----------------|-----------------|---------------------|
| MHC class II        |        |              |                |                 |                |                 |                     |
| pccs01.10/IEk       | B10.A, F1/A | 16   | 4,2,2,2  | 2,2,2 | Vα11 (15) | JαTA84 (10) | 9 (6) |
| SpWMb10-121/IEd     | DBA/2  | 6    | 3       | NF    | Vα1 (4) | JαC5 (3) | 10 (3) |
| MBP59.9k/IA*        | PL/J, (P x S) F1 | 8    | 3,2     | 2    | Vα4 (8) | JαTA31 (6) | 9 (6) |
|                     | B10.PL | 7    | 6       | 3    | Vα2 (6) | JαTA39 (6) | 8 (6) |
| αC12-26/IEk         | A/J    | 11   | 3,3     | 3    | Vα2 (11) | JαTA1 (7) | 8 (6) |
| HA110-120/IEd       | BALB/c | 13   | NF      | NF   | NF      | NF              |                   |
| MHC class I         |        |              |                |                 |                |                 |                     |
| PbCS252-260/Kd      | BALB/c, (B x C)F1 | 16   | NF      | NF   | NF      | NF              |                   |

For abbreviations and references, see Table 4. The Vα indicates subfamilies. As opposed to the β chain, the non-V, non-J-encoded region of the α chain is too small and unprecise to look for amino acid conservation.
substituted related peptides (Romero et al., manuscript in preparation). The clones thus appear to recognize a large number of epitopes on the CS nonapeptide-K^d complex. This heterogeneity of fine specificity patterns clearly correlates with the diversity of TCR primary structure found for this set of CTL clones specific for the P. berghei CS nonapeptide-K^d complex.

A Paradoxical V\beta Dominance. As mentioned previously, the dominance of the V\beta13 gene segment may be peptide related, at least in part, since other K^d-restricted responses apparently fail to show a V\beta13 dominance. In agreement, a similar V\beta dominance has been reported for the other responses studied, with no apparent correlation to the restriction element (Table 4). In the currently prevailing models of TCR-MHC-peptide interaction (3, 63, 64), the CDR-1-like and CDR2-like loops of both \alpha and \beta chains, encoded by the V\alpha and V\beta gene segments, respectively, are thought to interact with MHC residues, whereas the CDR3-like loops would interact with the peptide. This general topology was based on the much higher variability of the CDR3 loop and the peptide, when compared to CDR1 and CDR2 loops and the MHC molecule, respectively. It is now supported by increasing experimental evidence, showing that naturally occurring (14, 16, 18, 23, 27, 28) and experimentally engineered (65) TCRs that vary only in a CDR3 loop display distinct peptide fine specificities. Accordingly, an apparently peptide-related V\beta dominance in the context of otherwise highly diverse TCRs, such as we found in this study, is paradoxical. In the other studies, the overall diversity of the TCRs is very limited, thus, the V\beta dominance might be considered as an indirect consequence of a peptide-related constraint acting on other parts of the receptor, for example on the conserved amino acid in the CDR3 loop thought to be a peptide contact residue (16, 27, 65). In contrast, the high diversity of the TCRs in this study strongly suggests that the V\beta dominance in general might result from direct peptide-related constraints imposed by the peptide-MHC complex. Whether the V\beta dominance reflects a direct interaction of the V\beta segment with the peptide or an indirect effect of the bound peptide on the complex that in turn would favor the interaction of the V\beta segment with the restriction element is unknown.

What Determines the Size of the TCR Repertoire? Apart from the V\beta dominance, the extent of diversity of the T cell repertoire appears to vary considerably according to the MHC-peptide ligand involved. Why the CTL response to a P. berghei nonapeptide appears to be more diverse than the other reported T cell responses is unknown. It may be significant that the latter studies analyzed class II MHC-restricted T helper responses. This difference might allow a compensation for the apparently lower number of class I-than class II-restricted antigenic sites within proteins.

More likely, the size of the MHC-peptide complex–specific repertoire would depend primarily on the overlap between epitopes displayed by self peptides and the antigenic peptide bound to the same restriction element. This would explain why the responses towards polymorphic variants of self proteins (pcc, SpWMb) are so limited and the autoimmune response against MBP is oligoclonal. Even the Acl peptide, although of viral origin, has been shown by Gefter and colleagues (66) to be homologous to a self peptide able to bind the same restriction element. Conversely, responses to the viral HA determinant and the parasite CS peptide may be more diverse because relatively few self peptides would generate overlapping tolerogen epitopes. Although the CTL response to the whole P. berghei CS protein in H-2^K mice is focused primarily on a single nonapeptide in the context of H-2K^d, this peptide may be so distant to self that it triggers a highly diverse, and presumably highly potent, T cell response.

We thank J.-C. Cerottini for helpful discussions and his continuing interest and support. We are indebted to B. Malissen, M. Malissen, P. Marche, E. Jouvin-Marche, and A.-C. Garapin for helpful discussions and communication of unpublished results. We also acknowledge H. Acha-Orbea, G. Corradin, T. Crompton, G. Eberl, C. Jaulin, J. Kanellopoulos, T. Herrmann, H. R. MacDonald, B. Malissen, and C. Pannetier for helpful comments and critical reading of the manuscript. E. Mottez for oligonucleotides synthesis, and A.-S. Cordey, and K. Mühlethaler for superb technical assistance. J.-L. Casanova would like to express his gratitude to Steven E. Ross for his most helpful encouragement and support.

J.-L. Casanova was supported in part by the Institut National de la Santé et de la Recherche Médicale and in part by the European Organization for the Research on Treatment of Cancer. P. Romero was supported by the Roche Research Foundation.

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Received for publication 17 June 1991 and in revised form 12 August 1991.

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