Public and Private Vβ T Cell Receptor Repertoires Against Hen Egg White Lysozyme (HEL) in Nontransgenic Versus HEL Transgenic Mice

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Summary

We have previously produced a transgenic mouse line for hen egg lysozyme (HEL), an experimental model for analyzing tolerance to self-antigens at the peptide level. We have now characterized transgenic mice with HEL blood levels below 2 ng/ml, where significant T cell proliferative responses to HEL and its immunodominant peptide were observed. This HEL-low transgenic model was chosen because it mimics physiological conditions in which autoreactive T lymphocytes, recognizing self-components expressed at very low levels, persist without inducing a break in tolerance. Furthermore, in H-2d mice, HEL-specific T lymphocytes are triggered by a single immunodominant region, allowing us to compare the HEL-specific T cell Vβ repertoires of transgenic and nontransgenic animals against a single peptide presented as self or foreign, respectively. We found that a Vβ8.2-Dβ1-Jβ1.5 rearrangement is found in response to HEL in all nontransgenic mice, whereas this Vβ-restricted response is absent in HEL-low transgenic animals. At the nucleotide level, this rearrangement results from the trimming of the genomic segments during VDJ or DJ joining, without N additions, suggesting that the dominant rearrangement is selected early during fetal or neonatal life, before the expression of terminal deoxynucleotidyl transferase. In HEL-low transgenic mice, no dominant rearrangements are found as alternatives to the one observed in normal mice. Instead, each transgenic animal uses a different set of Vβ-Jβ combinations in its response to the immunodominant HEL peptide. In nontransgenic mice, besides the dominant Vβ8.2-Dβ1-Jβ1.5 combination, minor Vβ repertoires were found which differed in each animal and were distinct from the rearrangements used by individual transgenic mice. These findings suggest that the T cell response to an immunodominant peptide involves a “public” Vβ repertoire found in all animals and a “private” one which is specific to each individual.

The α/β TCR for antigen is a heterodimeric disulphide-linked membrane glycoprotein that specifically interacts with peptides bound to MHC molecules. It is composed of two polypeptide chains with variable (Vα and Vβ) and constant (Cα and Cβ) domains (1). The variable domains result from the somatic recombination of V and J gene segments for Vα and V, D, and J gene segments for Vβ. The diversity generated by these rearrangements is increased by imprecise joining of the various gene segments and addition of template-independent N nucleotides during this process (2).

T cell responses are induced by foreign antigens presented as peptides in the context of self-MHC molecules (3). A synthetic peptide can mimic a foreign antigen if it has the same amino acid sequence as the native epitope presented by MHC molecules (4, 5). It is now well established that foreign antigens as well as self-proteins are processed into peptides, which are loaded and presented by MHC molecules (6–11). Studies on TcR repertoires have shown that the Vα-Jα and Vβ-Jβ rearrangements, from antigen-specific TcRs, are selected by MHC-restricted antigenic peptides (12–15). In the thymus, immature lymphocytes go through complex steps of differentiation and selection that eliminate self-reactive cells and stimulate those that can recognize foreign peptides in the context of self-MHC (16–20).

Not all self-reactive T cells are eliminated. Autoreactive T cell responses have been reported in models of central as well as peripheral tolerance (21–27). Normally, self-proteins are processed in the thymic APCs in such a way that only self-peptides bound to MHC molecules in sufficient amounts can tolerize specific T lymphocytes. However, subdominant...
models of peripheral tolerance, extrathymic self-peptides and crypticity, see Sercarz et al. [28]). In transgenic mouse models of peripheral tolerance, extrathymic self-peptides presented by nonprofessional APCs lacking the appropriate accessory molecules can either be ignored by specific T lymphocytes (26) or lead to different states of energy of autoreactive T cells (27). But these self-reactive T cells can be activated and induce specific tissue destruction (27).

We have previously studied, in a mouse transgenic line for hen egg lysozyme (HEL), the state of tolerance to HEL and various peptides derived from it (24). We have found that H-2d transgenic mice were tolerant to HEL and its single immunodominant determinant. However, the state of tolerance to SD peptides 1-18 and 74-96 was highly dependent on HEL blood concentrations in transgenic animals. Autoreactive T lymphocytes specific for these SD peptides were inactivated as HEL serum level increased (24). Furthermore, HEL transgenic mice with HEL blood concentrations below 2 ng/ml (HEL-low transgenic mice) respond to HEL and to the ID 15-mer peptide 103-117 (29). It is interesting to note that the minimal ID peptide 108-116, which induces producible T cell proliferative responses in nontransgenic mice, does not elicit any response in most of the HEL-low transgenic mice tested. This suggests that, in these transgenic mice, the HEL-specific TCR repertoire has been negatively selected only partially since in vitro responses to the 15-mer ID peptide are still obtained. In another transgenic mouse model, human insulin is expressed at serum levels below 0.2 ng/ml and specific T cell proliferative responses are recalled in vitro by the human hormone (23).

Since HEL-specific T lymphocytes in H-2d mice are stimulated by only one ID region, we could compare the HEL-specific T cell Vβ repertoires of transgenic and nontransgenic animals against a single determinant presented as a self or as a foreign peptide, respectively. Using a new methodology described by Pannetier et al. (30), we studied the TcR Vβ repertoires of HEL-specific T lymphocytes in individual mice. We found that the most frequently selected TcR Vβ repertoire, Vβ8.2-Dβ1-β1.5, is observed in all nontransgenic mice, whereas it is lacking in HEL-low transgenic mice. The Vβ repertoire in HEL-low transgenic mice is striking, since each animal uses a different set of Vβ-Jβ rearrangement, whereas the minor combinations are different in each mouse and may correspond to the only response seen in the transgenic mice.

Materials and Methods

Mice. Transgenic mice for HEL have been described elsewhere (24). Transgenic mice, nontransgenic littersmates, and control mice (BALB/c, H-2d) were bred and maintained at the Centre de Service et d'Elevage des Animaux de Laboratoire (CSEAL) (Centre National de la Recherche Scientifique, Orléans, France) in specific pathogen-free conditions. A standard PCR assay with HEL primers was used as a fast screen for transgenic mice. HEL serum concentration in transgenic mice was measured by an ELISA assay described previously (24).

Proliferation Assay. Transgenic and control mice were immunized in the hind footpads with 3.5 nmol of HEL in CFA. 9 d later, popliteal lymph nodes were collected, and lymph node cells (LNC) were cultured at 5 x 10⁵ cells per well with different concentrations of HEL or 15-mer 103-117 peptide and 5% CO₂ for 4 d. All cultures were done in HEL-L medium (Ventrex Laboratories, Portland, ME) supplemented with glutamine (2 mM). Then, stimulated cells were collected in order to extract their mRNA or to produce T cell hybridomas.

T Cell Hybridomas. Lymphocytes stimulated 4 d in culture by HEL were collected and mixed with the thymoma BW5147 (TCR-α/β-) (31) at a 4:1 ratio in a solution (50:50, vol/vol) of PEG (poly-ethylene-glycol, hybrimax, Sigma Chemical Co., St. Louis, MO) and RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) prewarmed at 37°C for 3 min. The cells were washed once and resuspended in RPMI 1640 medium supplemented with glutamine (2 mM), 2-ME (5 x 10⁻⁵ M), and 20% FCS (Serovial, Brumath, France) and cultured at a concentration of 2 x 10⁵ cells per well in 96-well plates (Falcon Labware, Oxnard, CA). A solution of HAT was added to each well and the plates were incubated at 37°C in 5% CO₂. 1-2 wk later, T cell hybridomas were tested for their reactivity to HEL and ID peptide 103-117 by measuring the amount of IL-2 released in 24 h. This was done by the colorimetric method described by Mosmann (32). The cells from positive wells were then cloned at 0.3 cells per well.

Oligonucleotides. Sense oligonucleotides specific for each of the 23 Vβ chains and antisense oligonucleotides for Cβ and Jβ were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, CA). These oligonucleotides have been described elsewhere (30). Fluorophores were attached via an aminolink-2 at the 5' end of the β8 oligonucleotide, Vβ3, Vβ5, Vβ11, and Vβ17 were not measured since these Vβ8s are deleted by mouse mammary tumour virus (MMTV) in BALB/c mice (33).

mRNA Extraction and cDNA Synthesis. mRNAs from T cell hybridomas and lymphocytes in culture were extracted with a Quick mRNA Micro Prep Kit (Pharmacia, Piscataway, NJ). Briefly, cells were lysed in guanidium thiocyanate and mRNAs were purified by affinity chromatography on oligo-dT cellulose. After ethanol precipitation, mRNAs were dissolve in water, aliquoted, and kept at -80°C. mRNAs were reverse transcribed into cDNA using a cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). After denaturation at 70°C for 10 min, mRNAs were incubated with poly-dT primers (5 μM) and 2 U of reverse transcriptase (from AMV) at 43°C for 1 h, followed by an incubation at 53°C for 10 min. A PCR assay was done with aldolase primers in order to control each cDNA preparation.

PCR and Run-off Reactions. For PCR, aliquots of cDNA were...
amplified in a 50-μl reaction volume with the given Vβ and Cβ primers using 2 U of Taq DNA polymerase (Promega, Madison, WI) in the supplier buffer. 40 cycles were performed, involving first a 1-min denaturation step at 94°C, a 1-min annealing step at 60°C, and a 4-min polymerization step at 72°C, followed by an elongation step at 72°C for 10 min. For all PCRs performed, a negative control was included where cDNA was replaced by water. PCR products were electrophoresed, precipitated, and dissolved in water before use in sequencing or run-off reactions.

For run-off reactions and determination of relative index of stimulation (RIS), PCR products were elongated with each of the 12 fluorophore-labeled Jβ oligonucleotides in a five cycle PCR step (run-off) and loaded on a 6% acrylamide sequencing gel on a DNA sequencer (model 373A; Applied Biosystems). For each Vβ-Jβ rearrangement, the fluorescent run-off products of various sizes that had been elongated through the CDR3 regions were separated on the denaturing gel and their lengths calculated with appropriate size standards. In all Vβ-Jβ combinations, these elongation products were distributed into 6–11 peaks and corresponded to in-frame transcripts. For any given peak, after treatment of the data by software developed in our laboratory (30), the RIS was calculated using the following formula: RIS = [(area of one experimental peak)/(sum of areas of all other experimental peaks)/ (area of corresponding control peak)/(sum of areas of all other control peaks)]. This equation can be written: RIS = [(area of one experimental peak)/(area of corresponding control peak) x (sum of areas of all other control peaks)/(sum of areas of all other experimental peaks)] (1). When the peak profiles from the second term of the equation (1) were fitted so as to maximize the number of superimposed peaks, the second term is about 1. Thus, after this normalization, a simpler formula of the RIS was obtained: RIS = (area of one experimental peak/area of corresponding control peak). Therefore, an increase in RIS for a peak of a given nucleotide length is directly proportional to the augmentation of KNA messengers corresponding to this particular Vβ-Jβ rearrangement.

Sequence of PCR Products. PCR products were directly sequenced using the method described by Casanova et al. (34). Briefly, aliquots of PCR products were denatured by boiling at 100°C for 10 min in the presence of a given primer and transferred quickly at −70°C in dry ice and ethanol (95%). The frozen material was simultaneously thawed and mixed with [35S]dATP and sequencing kit (United States Biochemical Corp., Cleveland, OH) for 45 s in the supplier buffer. The dNTPs were then added and the elongation was performed for 3 min at room temperature. The material was then loaded on a 6% acrylamide sequencing gel. Autoradiography of the dried gel was performed at room temperature with Kodak films.

Results

Choice of the Transgenic Animals and of the HEL Peptide. We have previously produced a HEL transgenic mouse line by microinjecting (BALB/c × DBA/2) × BALB/c eggs (H-2d haplotype) with a HEL cDNA placed under the control of the ubiquitously expressed housekeeping gene promoter, the mouse hydroxymethyl glutaryl Co-A reductase (24). The transgenic line was derived from one female founder subsequently backcrossed to BALB/c males. The repertoire studies presented here were performed with transgenic and nontransgenic animals from crosses between HEL transgenic males and BALB/c females, in order to avoid a potential tolerization of nontransgenic littersmates by transplacental influx of HEL from their transgenic mother. Such a maternal effect has been observed previously in nontransgenic mice born from females transgenic for the secreted hepatitis B antigen (35).

HEL transgenic mice express different amounts of HEL, even in the same litter, but in a given individual, HEL blood level variations do not exceed ± 30%. These features of the HEL transgenic line have allowed us to study the state of tolerance to HEL in transgenic animals over a broad range of HEL blood concentrations (0.5–1,500 ng/ml). Our previous results have shown that mice expressing >10 ng/ml are fully tolerant to HEL. However, mice expressing <2 ng/ml often display T cell proliferative responses when immunized with HEL (29). These HEL-low transgenic mice were further analyzed with HEL peptides in vitro recall experiments: LNC from HEL-low animals immunized with HEL in CFA were incubated with various HEL peptides. The 15-mer ID peptide 103-117 triggered only a proliferative response, whereas the 9-mer ID peptide 108-116 and SD peptides 1-18 and 74-96 had no effect. Nontransgenic BALB/c mice respond to both ID peptides 108-116 and 103-117. Hence, we could analyze and compare the T cell responses to the ID 103-117 peptide in BALB/c nontransgenic mice and in partially tolerant HEL-low transgenic mice. For a variety of reasons discussed below, our analysis was focused on the β chains of TcR reacting with the ID 103-117 peptide.

Analysis of a Hybridoma Collection. We had previously described a collection of 14 T cell hybridomas that respond to HEL and to the 103-117 peptide (29). These were isolated from HEL-primed LNC of BALB/c mice triggered in vitro by HEL for 4 d. As a first approach to our repertoire analysis, we decided to sequence the TCR β chain transcripts of these hybridomas. For this, we extracted RNA from each hybridoma, prepared cDNA, and performed a PCR with sense oligonucleotides specific for each of the 23 known Vβ segments and antisense Cβ oligonucleotide (30). We then sequenced the double-stranded PCR products (34). All nucleotide sequences contained open reading frames encoding TcR Vβ residues. The expression of Vβ8.2 and Vβ6 channels was confirmed by flow cytometry analysis of the T cell hybridomas after staining with the corresponding anti-Vβ mAbs (data not shown). The Vβ8.2 gene segment is expressed in 12 out of 14 T cell hybridomas (Fig. 1). Among the 12 Vβ8.2 T cell hybridomas, 6 different Jβ segments were rearranged (Jβ1.1, Jβ1.4, Jβ1.6, Jβ2.1, Jβ2.3, and Jβ2.4). Four CDR3 of different lengths (8, 9, 10, and 13 amino acids), as defined by Chothia et al. (36), were observed. Although the 12 TCR Vβ junctional regions were very diverse in terms of Jβ segment usage and CDR3 length, four amino acid residues of the CDR3 loop were highly conserved: G95, T96, G97, and N98 (Fig. 2). Furthermore, Q98, a residue mostly encoded by HEL for 4 d. As a first approach to our repertoire analysis, we decided to sequence the TCR β chain transcripts of these hybridomas. For this, we extracted RNA from each hybridoma, prepared cDNA, and performed a PCR with sense oligonucleotides specific for each of the 23 known Vβ segments and antisense Cβ oligonucleotide (30). We then sequenced the double-stranded PCR products (34). All nucleotide sequences contained open reading frames encoding TcR Vβ residues. The expression of Vβ8.2 and Vβ6 channels was confirmed by flow cytometry analysis of the T cell hybridomas after staining with the corresponding anti-Vβ mAbs (data not shown). The Vβ8.2 gene segment is expressed in 12 out of 14 T cell hybridomas (Fig. 1). Among the 12 Vβ8.2 T cell hybridomas, 6 different Jβ segments were rearranged (Jβ1.1, Jβ1.4, Jβ1.6, Jβ2.1, Jβ2.3, and Jβ2.4). Four CDR3 of different lengths (8, 9, 10, and 13 amino acids), as defined by Chothia et al. (36), were observed. Although the 12 TCR Vβ junctional regions were very diverse in terms of Jβ segment usage and CDR3 length, four amino acid residues of the CDR3 loop were highly conserved: G95, T96, G97, and N98 (Fig. 2). Furthermore, Q98, a residue mostly encoded in the N diversity region, is a close homologue of N98 and is found in four independent T cell hybridomas. Finally, C6.2 and M3.13, two independent hybridomas using Vβ8.2, Dβ1, and Jβ1.5 segments, displayed an identical CDR3 sequence: G95 T96 G97 N98 N99 Q100 A101 P102. These results provide a useful database but, in terms of
reertoire analysis, the hybridoma approach suffers severe limitations: it is quite labor intensive, and it is difficult to isolate hybridomas in large enough numbers that would make an analysis of the minor responses statistically significant. Furthermore, the distribution of TCR sequences in hybridomas might not reflect accurately the in vivo situation. We, therefore, turned to a PCR-based approach, which provides a global description of the β chain transcripts and can be applied to individual mice.

**Distribution of TCR β Chain Transcripts in BALB/c Mice Immunized with HEL.** We first explored the in vivo situation in BALB/c mice immunized with HEL. The experimental approach has been described in detail elsewhere (30). Briefly, RNA extracted from a mixture of 5 × 10^6 to 5 × 10^7 LNC al~ was reverse transcribed into cDNA, and aliquots were amplified by PCR, using every one of the V~β-specific oligonucleotide primers and one C/β-specific primer. The product of each PCR was then divided into 12 aliquots that were hybridized with a fluorescein-labeled oligonucleotide specific for each of the 12 known J/β segments. A run-off reaction was performed, and the sizes of the fluorescent run-off products were analyzed in an automated DNA sequencer. In each run-off reaction, the polymerase transcribed the CDR3-like regions of various sizes, which were quantitatively recorded in the run-off products. For any given V/β-J/β combination, a set of about seven size classes, spaced by three nucleotides and corresponding to in-frame transcripts, was usually observed. A sample of the actual graphical display is shown in Fig. 3. The area under each peak is proportional to the number of elongated molecules sharing the same CDR3 length. Leaving out V/β which are deleted by MMTV in BALB/c mice, there are 18 functional V/βs (33). A complete analysis, therefore, involves 18 × 12 = 216 run-off reactions, each yielding an average of seven peaks of different sizes, such that about 1,500 peaks are thus visualized. Each of these includes an unknown number of transcripts (perhaps dozens or hundreds) sharing the same V/β, the same J/β, and the same CDR3 length, but having distinct CDR3 sequences.
We know from previous work (30) that the Gaussian-like distribution of the peaks is highly reproducible and that a significant perturbation of this distribution can be observed for specific combinations of Vβ, Jβ, and CDR3 size, upon immunization with a protein antigen. Thus, the area of the rearrangement length in nucleotides highest peak for each Jβ. To have a clearer view, the control profiles (----) were slightly shifted to the right of those observed with run-off products from HEL-stimulated lymphocytes (----).

BALB/c mice were immunized with HEL in CFA (once or twice), BALB/c mice injected with CFA alone served as controls, and popliteal or inguinal LN RNAs were extracted and analyzed as described above. Because most of the hybridomas used the Vβ8.2 gene segment, we scanned Vβ8.2 run-off products combined with each Jβ segment. In Fig. 3 A, we show peaks from the same mouse immunized twice. A major alteration is seen in the Vβ8.2-Jβ1.5 combination, where a peak corresponding to a CDR3 size of eight amino acids emerges with a RIS of 3. The size of this CDR3 is precisely that found in the three independent T cell hybridomas specific for the HEL peptide 103-117 restricted by I-Ea and utilizing Vβ8.2 and Jβ1.5 segments, as described in Fig. 1. It is worth noting that, although four hybridomas shared Vβ8.2 and Jβ2.3, with CDR3 lengths of eight and nine amino acids, respectively (D96 and A97), in summary, the results indicate that an vivo T cell response to lysozyme can be detected by our approach, and that it fitted, but only to a limited extent, the rearrangement found in our hybridoma collection.

Extended Analysis of TcR β Chain Transcripts in BALB/c Mice Immunized with HEL or Peptide 103-117. We then focused our analysis on the ID peptide 103-117 in the following way: mice were injected with 7 nmol of HEL in CFA, and 9 d later, their LNC were recalled in vitro with 14 nmol of HEL or peptide 103-117 for 4 d, after which RNA was extracted and analyzed as above. 10 individual mice were ana-

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**Figure 3.** (A) Profiles of the fluorescent Vβ8.2-Jβ8 run-off products obtained with LNC of two nontransgenic mice. One was immunized with HEL in CFA and boosted 15 d later with HEL in IFA (----), the second one (control) with adjuvants only (---). LNC RNAs were extracted 9 d after the boost. The intensity of the fluorescence is represented in arbitrary units as a function of the size of the single-stranded DNA fragments. RIS is presented between parentheses next to a few peaks. (B) Profiles of the fluorescent Vβ8.2-Jβ8 run-off products obtained from HEL primed LNC of nontransgenic mice No. 29, stimulated in vitro with HEL or peptide 103-117 (----) or from LNC of nontransgenic mice immunized with CFA and stimulated in vitro with PPD as control (----). In A and B, the HEL and CFA profiles were superimposed and normalized with respect to the highest peak for each Jβ. To have a clearer view, the control profiles (----) were slightly shifted to the right of those observed with run-off products from HEL-stimulated lymphocytes (----).

**Figure 4.** Nucleotide and deduced amino acid sequences of the junctional regions of the Vβ8.2-Jβ1.5 rearrangements found in LNC from nontransgenic mice immunized twice with HEL as described in Fig. 3. RNAs were prepared, reverse transcribed, and the cDNAs were extracted and analyzed as described above. Because most of the hybridomas used the Vβ8.2 gene segment, we scanned Vβ8.2 run-off products combined with each Jβ segment. In Fig. 3 A, we show peaks from the same mouse immunized twice. A major alteration is seen in the Vβ8.2-Jβ1.5 combination, where a peak corresponding to a CDR3 size of eight amino acids emerges with a RIS of 3. The size of this CDR3 is precisely that found in the three independent T cell hybridomas specific for the HEL peptide 103-117 restricted by I-Ea and utilizing Vβ8.2 and Jβ1.5 segments, as described in Fig. 1. It is worth noting that, although four hybridomas shared Vβ8.2 and Jβ2.3, with CDR3 lengths of eight and nine amino acids, the corresponding peaks did not show a significant increase in RIS values in vivo. Nine individual mice were analyzed in this way, and the results were highly reproducible: every mouse displayed a major Vβ8.2-Jβ1.5 peak (i.e., having the highest RIS) with a CDR3 size of eight amino acids. We sequenced three TcR corresponding to peaks that displayed the highest RIS values (Fig. 4). One of them shares the amino acid CDR3 sequence with the C6.2 and M3.13 T cell hybridomas. The other two differ by only one and two amino acids, respectively (D96 and A97). In summary, the results indicate that an vivo T cell response to lysozyme can be detected by our approach, and that it fitted, but only to a limited extent, the rearrangement found in our hybridoma collection.
### PANEL A

| NTG | vβ | FW CDR3 | FW | Jβ |
|-----|----|---------|----|----|
| 29  | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 46  | 8.2 | TGT GCC AGC GGT ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 38  | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 59  | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 241 | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 243 | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 244 | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 1   | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 2   | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 3   | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 4   | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 5   | 8.2 | TGT GCC AGC GGG.ACA..GGG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
|     | 8.2 | C A S G T G Q T N E R L F F G |    |    |
| 9   | 8.2 | TGT GCC AGC GGT ACT.GGA_CAG.ACC AAC GAA AGA TTA | TTT TTC GGT | 1.4 |
| 244 | 4   | TGT GCC AGC AGC CAT.CCC.CCG..GGA..CAG..GCT AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |

### PANEL B

| TQ | vβ | FW CDR3 | FW | Jβ |
|----|----|---------|----|----|
| 23 | 8.3 | TGT GCC AGC AGT GAT GGA..CAA..AAT TCC AAC GAA AGA TTA | TTT TTC GGT | 1.4 |
| 25 | 1   | TGT GCC AGC CAA.GAT.AAG..ACA..ACA GAA ACG CTG | TAT TTT GGC | 2.3 |
| 33 | 8.3 | TGT GCC AGC AGT GAG..GGA..CAG AAC AAC CAG GCT CCG | CTT TTT GGA | 1.5 |
| 34 | 15  | TGT GCT GCT..AGG..GGA..CAA AAG ACC ACC TTAG | TAC TTT GGT | 2.4 |

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lyzed for Vβ8.2 and all Jβs. In addition, four individual mice were analyzed for the 18 Vβs (except Vβ8.1, which failed for technical reasons) and all Jβs.

A sample of the results is shown in Fig. 3 B. The predominant change in the Vβ8.2-Jβ1.5 major rearrangement is now more pronounced, with a RIS of 6.5 after in vitro recall. This confirmed our expectation that the response observed in vivo against HEL was indeed directed against the ID peptide 103-117. Again, this result was highly reproducible, and the major Vβ8.2-Jβ1.5 rearrangement with a CDR3 size of eight amino acids was observed in all 14 mice with RIS values ranging from 5 to 12.

We then sequenced the Vβ8.2-Jβ1.5 PCR product in 12 cases and obtained a readable sequence, proving the clonal character of the amplified product (Fig. 5 A). 11 of the 12 sequences were identical to that of the C6.2 and M3.13 T cell hybridomas. The remaining one differed by a single, silent nucleotide change. We ruled out the possibility that these sequences could have originated from some contamination during the PCR because: (a) all sequences were not determined simultaneously; (b) negative controls were added to each experiment showing that reagents were clean; (c) cDNAs from protein purified derivative (PPD)-stimulated LNC, when processed at the same time as cDNAs from 103-117 peptide-specific T cells, never showed such a dominant sequence; and (d) in all HEL-low transgenic mice tested, we never found this dominant rearrangement.

The Vβ8.2-Jβ1.5 (CDR3 length = 8) was not the only peak that emerged in BALB/c mice after immunization with HEL and in vitro recall with HEL or peptide 103-117. Given the high reproducibility of the Gaussian-like distribution of the peak sizes in this and other studies, we estimated that RIS values >4 are highly characteristic of the presence of a single expanded T cell clone. The data obtained with the four individual mice for which the entire repertoire was scanned, are summarized in Table 1, where all peaks displaying a RIS values >4 in the other Vβ-Jβ combinations. The results are summarized in Table 1. Peaks with high RIS show up in variable numbers and distinct combinations in individual mice. Two transgenic mice, 23 and 33, preferentially use Vβ8.3 in association with different Jβ segments, Jβ1.4 and Jβ1.5, respectively. The other two use a larger Vβ repertoire. These minor Vβ-Jβ combinations (found in mice Nos. 25 and 34, Table 1) are heterogeneous; eight different Vβs are used between the two mice with only one Vβ-Jβ in common, Vβ6-Jβ2.7. Among the Vβ6-Jβ combinations used, none was similar to the two Vβ6-Jβ chains of HEL-specific T cell hybridomas C5.4 and D12.8. The rearrangements with the highest RIS values found in each transgenic mouse were sequenced. In some instances, readable nucleotide CDR3 sequences were obtained, and in-frame amino acid sequences were deduced as presented in Fig. 5 B. In three out of four sequences, we observed three conserved residues in the CDR3: G, Q, and N. These amino acids were found as GQN or GQQN motifs in different positions in CDR3 sequences. It is interesting to note that these CDR3 motifs are reminiscent of the GNNQ sequence found in the CDR3 of the dominant Vβ8.2-Jβ1.5 rearrangement of HEL-specific nontransgenic T cells.

The T cell Vβ repertoires from transgenic and nontransgenic mice showed that only a few Vβ-Jβ were shared by both groups of mice: Vβ1-Jβ2.3 (mice Nos. 25 and 243), Vβ4-Jβ1.6 (mice Nos. 34 and 241), and Vβ13-Jβ2.3 (mice Nos. 34 and 29). We could not compare the sequences from these rearrangements since only the CDR3 sequence of Vβ1-Jβ2.3 from mouse No. 25 was readable on a sequencing gel. It is striking that when the dominant rearrangement is not used, HEL-specific T cells from transgenic or nontransgenic animals express very different Vβ-Jβ combinations. In HEL-low transgenic mice, no minor rearrangement becomes dominant.

Analysis of HEL-low Transgenic Mice. Four transgenic animals of the HEL-low type were analyzed in detail as above. A major rearrangement of the Vβ8.2-Jβ1.5 (CDR3 length = 8) type could not be detected in any of the mice. To confirm this, we sequenced the Vβ8.2-Jβ1.5 PCR products and, in contrast to the nontransgenic situation, we could not obtain a readable sequence. We then searched for peaks with RIS values >4 in the other Vβ-Jβ combinations. The results are summarized in Table 1. Peaks with high RIS show up in variable numbers and distinct combinations in individual mice. Two transgenic mice, 23 and 33, preferentially use Vβ8.3 in association with different Jβ segments, Jβ1.4 and Jβ1.5, respectively. The other two use a larger Vβ repertoire. These minor Vβ-Jβ combinations (found in mice Nos. 25 and 34, Table 1) are heterogeneous; eight different Vβs are used between the two mice with only one Vβ-Jβ in common, Vβ6-Jβ2.7. Among the Vβ6-Jβ combinations used, none was similar to the two Vβ6-Jβ chains of HEL-specific T cell hybridomas C5.4 and D12.8. The rearrangements with the highest RIS values found in each transgenic mouse were sequenced. In some instances, readable nucleotide CDR3 sequences were obtained, and in-frame amino acid sequences were deduced as presented in Fig. 5 B. In three out of four sequences, we observed three conserved residues in the CDR3: G, Q, and N. These amino acids were found as GQN or GQQN motifs in different positions in CDR3 sequences. It is interesting to note that these CDR3 motifs are reminiscent of the GNNQ sequence found in the CDR3 of the dominant Vβ8.2-Jβ1.5 rearrangement of HEL-specific nontransgenic T cells.

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Figure 5. (A) Nucleotide and deduced amino acid sequences of junctional regions of the dominant Vβ8.2-Dβ1-Jβ1.5 rearrangement found in different nontransgenic mice (Nos. 29-244) and BALB/c mice (Nos. 1-5) and of minor rearrangements (Vβ8.2-Jβ1.4 and Vβ4-Jβ1.5) found in BALB/c mouse No. 3 and nontransgenic No. 244, respectively. Mice were immunized with HEL in CFA. 9 d later, the LNC were collected and stimulated in vitro with HEL or peptide 103-117 for 4 d. LNC RNAs from both types of cultures were prepared and reverse transcribed. cDNAs were amplified with sense Vβ8.2 and antisense Jβ1.5 primers and the amplified products were sequenced. All nucleotide sequences of dominant rearrangements were identical except for mouse No. 46 where a G in the 4th codon was replaced by a T. In this case, the Dβ1 segment used is one codon shorter, the latter being replaced by the codon GGT from the Vβ8.2 segment. The nucleotides of Dβ1 origin are underlined (....) as are N diversity-encoded nucleotides (___). (B) Nucleotide and deduced amino acid sequences of major peaks found in HEL-specific T lymphocytes from individual HEL-low-transgenic mice. Underlined sequences correspond to Dβ1 (___) or N diversity-encoded nucleotides (___). These sequence data are available from EMBL/GenBank/DDBJ under accession number X79967-X79973.
## Table 1. Vβ:Jβ Rearrangements Observed in HEL-specific T Lymphocytes from Nontransgenic and HEL-low Transgenic Mice

| Transgenics | Nontransgenic |
|-------------|---------------|
| N° | Vβ | Jβ | CDR3 length | RIS | N° | Vβ | Jβ | CDR3 length | RIS |
| 23 | 8.3 | 1.4 | 11 | 8.5 | 29 | 1 | 2.1 | 11 | 4.9 |
| 25 | 1 | 2.3 | 9 | 6.9 | 8.2 | 1.5 | 8 | 6.5 |
| 6 | 1.4 | 10 | 4.5 | 13 | 1.2 | 8 | 4.3 |
| 6 | 1.4 | 11 | 4.2 | 13 | 2.1 | 8 | 4.3 |
| 6 | 2.1 | 11 | 6.3 | 13 | 2.3 | 9 | 3.6 |
| 6 | 2.7 | 8 | 5.3 | 16 | 1.2 | 10 | 4.3 |
| 8.3 | 1.5 | 9 | 4.4 | 12 | 1.2 | 7 | 4.2 |
| 12 | 1.2 | 8 | 4.4 | 241 | 4 | 1.6 | 11 | 13 |
| 33 | 8.3 | 1.5 | 10 | 7.5 | 4 | 2.7 | 8 | 4.1 |
| 8.3 | 2.2 | 10 | 4.7 | 8.2 | 1.5 | 8 | 9.6 |
| 8.2 | 1.6 | 9 | 8 |
| 34 | 4 | 1.6 | 11 | 4.2 | 15 | 1.5 | 9 | 5.6 |
| 6 | 1.5 | 7 | 4.3 | 12 | 2.3 | 10 | 4.2 | 8.2 | 1.5 | 8 | 10 |
| 13 | 2.3 | 8 | 4.1 | 15 | 2.4 | 7 | 5.8 | 244 | 4 | 1.5 | 12 | 21 |
| 8.2 | 1.4 | 9 | 9.9 |
| 8.2 | 1.5 | 8 | 8.6 |

Mice were immunized with HEL in CFA. 9 d later, the draining LN were collected and LNC were stimulated in vitro with HEL or peptide 103-117. After 4 d in culture, RNAs from 10⁷ LNC were extracted and reverse transcribed. After amplification of the cDNA with the Cγ and 18 Vγ primers, the run-off reactions corresponding to each Vβ were done with the 12 fluorescent Jγ primers. For each mouse, 216 combinations were tested. CDR3 lengths are given for each peak that had a significant RIS value (RIS >4). The Vγ3, Vγ5, Vβ11, and Vβ17 were not tested since these VBs are deleted in BALB/c mice; the Vβ8.1 primer was not used in these experiments. **(Bold-face)** Rearrangements that gave readable nucleotide sequences.

## Discussion

We report here a detailed analysis of the T cell response of BALB/c mice and HEL-low transgenic mice against the ID peptide 103-117 of HEL. The HEL-low transgenic mice are partially tolerant to HEL. In recent work, we have shown that LNC from transgenic mice which have HEL blood levels <2 ng/ml can be recalled in vitro by HEL and by the ID peptide 103-117 but not by the minimal ID peptide 108-116 (29).

We first determined the nucleotide sequences of the transcripts encoding the TcR β chains of 14 T cell hybridomas directed against this dominant epitope. We then used a PCR-based approach to analyze the distribution of TcR β transcripts in LNC of BALB/c mice immunized with HEL, as well as in the LNC of BALB/c mice and HEL-low transgenic animals immunized once with HEL and recalled in vitro with HEL or the ID peptide 103-117. The TcR α chains were not studied by our method for two reasons. First, the multiplicity of Vα and Jα segments makes the analysis of α chain transcripts complex and difficult. Second, it is now well documented that T cells often display two in-phase α chain transcripts, which can obscure interpretations (38).

In the hybridoma collection, 12 out of 14 transcripts use the Vβ8.2 gene segment. Among these, three use Jβ1.5 and 4 Jβ2.3. The Vβ8.2-Jβ1.5 combination, with a CDR3 length of eight amino acids, emerged with a remarkable reproducibility in 9 out of 9 BALB/c mice in vivo, as well as after in vitro recall of LNC from 14 animals out of 14. Nucleotide sequences showed perfect or nearly perfect identity with two of the hybridoma sequences, C6.2 and M3.13. In contrast, the Vβ8.2-Jβ2.3 combination with CDR3 lengths of eight or nine amino acids, was undetected in vivo or upon recall in vitro. Most of the other combinations found in the hybridoma collection were also not detected, except for Vβ8.2-Jβ1.4 which was found in one case in vitro. This rearrangement was sequenced and the deduced amino acid sequence was very close to that of the Bl1.1 hybridoma which uses Vβ8.2-Jβ1.4 (Fig. 4). Thus, as far as the dominance of the
T cell response is concerned, for HEL (and contrary to cytochrome c [37]) the picture provided by the hybridoma collection is not a fully accurate reflection of the in vivo situation.

In LNC isolated from animals primed with HEL, the in vitro recall with an ID peptide tends to increase the sensitivity of detection of responsive T cell clones, since higher RIS for the Vβ8.2-Jβ1.5 combination are reproducibly observed with recalled LNC from nontransgenic animals. Thus, in all these animals that produce a highly Vβ-restricted response, there appears to be a predominant way to recognize the ID peptide in the T cell repertoire. It is worth noting that other combinations also emerge as peaks with elevated RIS, but, as summarized in Table 1, these peaks are different for each animal. In HEL-low transgenic animals, the dominant Vβ8.2-Jβ1.5 combination could not be observed, but peaks with high RIS were detected. They were different from one transgenic animal to the other, and were different also from those found in nontransgenic mice. It is important to emphasize that: (a) these peaks are not found in CFA-treated controls; (b) they are detected over otherwise highly reproducible patterns (the Gaussian-like distribution which is consistently observed in our hands); and (c) several peaks could be sequenced and some display a GQQN motif highly reminiscent of sequences found in the hybridoma collection. Therefore, these peaks cannot be the result of technical artefacts such as PCR contaminations or statistical fluctuation of the size distribution. We conclude that they do in fact represent true T cell responses to the ID peptide.

Poindexter et al. (39) have compared the repertoire of insulin-specific T cell hybridomas from normal and transgenic mice for human insulin. Their results showed that the Vβ repertoire of these T cell hybridomas is rather heterogeneous, with a predominant usage of Vβ1 in normal mice. In transgenic mice, the Vβ1 chain was utilized in one out of 16 hybridomas, suggesting that human insulin–specific T lymphocytes bearing Vβ1 TcR were deleted or anergized. Our findings are in agreement with such observations and extend the analysis by describing the full Vβ repertoire used by HEL-low transgenic mice in response to a single, well-defined ID peptide.

Our results can be summarized as follows. In BALB/c mice, the T lymphocytes that recognize in vitro the HEL ID peptide fall into two categories: a dominant clone which emerges reproducibly in all animals, and a series of clones which vary in number and sequence in distinct animals. This phenomenon can be described by assuming that the T cell response to the ID HEL peptide in BALB/c mice includes two components: a public response that is identical in all animals and a private response that is specific to each individual. According to this terminology, the hybridoma collection displays both public and private specificities. The public response is most likely directed against residues from the core determinant 108-116 of the ID peptide. The TcR(s) recognizing this core area presented by I-E<sup>d</sup> have the highest affinities, whereas the private responses may focus on residues from the longer peptide 103-117 or on additional residues only. In particular, one T cell hybridoma, G8.25, reacts strongly with peptide 103-117 but not with peptide 108-116, showing that at least some clones recognize a different epitope on the same peptide (29). It is striking that the HEL-low transgenic mice display the private but not the public arm of the response. We propose, therefore, that the high affinity specific clones recognizing the 108-116 I-E<sup>d</sup> complexes can be negatively selected in the presence of low levels of HEL. Thus, the high affinity Vβ8.2-Jβ1.5 clones are eliminated or inactivated. In HEL-low transgenic mice, residual proliferative responses are due to private clones which may have a lower affinity and/or focus on different residues of the ID peptide (such as hybridoma G8.25).

Do T cell responses, in general, include a public and a private component? The available published data are scarce but they suggest that this may indeed be the case. In B10.A mice immunized with pigeon cytochrome c (PCC) and recalled in vitro by the same protein, the Vβ3-Jβ1.2, Vβ3-Jβ2.5, and Vβ16-Jβ1.2 TcR rearrangements have been frequently selected by the PCC peptide 81-104 restricted by I-E<sup>b</sup> (13). Nevertheless, only the Vβ3-Jβ1.2 and, to a lesser extent, the Vβ3-Jβ2.5 rearrangement is observed in vivo (37). In an extensive study of the CTL response against the HLA-Cw3 peptide 170-179 presented by K<sub>d</sub> in DBA/2 mice, the TcR chains display very limited heterogeneity, both in terms of V<sub>α</sub>, J<sub>α</sub> and V<sub>β</sub>, J<sub>β</sub> segments in vitro. The dominant Vβ10-Jβ1.2 rearrangement observed in one out of two clones in vitro is always observed in vivo when mice are immunized with the HLA-Cw3 peptide (40). Similar observations were made by Levraud, J. P., C. Pannetier, P. Langlade-Demoyen, and P. Kourilsky (personal communication), who studied the Vβ repertoire of T lymphocytes infiltrating P815 mastocytoma tumors. A dominant Vβ1-Jβ1.2 rearrangement was found with a unique CDR3 amino acid sequence. This sequence was also found in two out of five CTL clones specific for a peptide derived from the P815 tumor-specific antigen, P1A. The three other CTL clones display rearrangements not found in vivo. Therefore, in three different experimental systems, a public response has been documented, but in all cases, hybridoma and/or clones with the same specificity contain rearrangements and/or sequences that were not found in vivo, and could well correspond to private specificities.

Is there any structural difference between public and private T cell clones? The dominant Vβ8.2-Jβ1.5 public rearrangement involves no N additions, whereas most of the private rearrangements do (Fig. 2). This absence of N additions is reminiscent of fetal or neonatal combinations, and suggests that they might have occurred before the onset of expression of terminal deoxynucleotidyl transferase. However, this rearrangement cannot be considered as strictly germline since the Vβ8.2, DJβ1.1, and Jβ1.5 genomic segments have been trimmed. The absence of N additions cannot, however, be a general feature of dominant public rearrangements since, in the case of Th1 response against PCC in B10.A mice (13, 37), and of the CTL response against the HLA-Cw3 peptide presented by K<sub>d</sub> (40), N additions are found. On the other hand, the CDR3 motif GQQ or GQQQ, shared by the private Vβ8.3-Jβ1.4, Vβ8.3-Jβ1.5, and Vβ15-Jβ2.4 rearrange-
ments, is homologous to the GNNQ sequence, which is found in the public dominant V\beta 8.2-J\beta 1.5 rearrangement. The presence of these residues is particularly striking since they result from the rearrangements of different V\beta and J\beta segments selected by the same peptide.

How do the private T cell clones originate, and how do they escape tolerization in the HEL-low transgenics? Two different explanations could be proposed to account for this observation. One hypothesis would be that the high-avidity public and private clones have been tolerated, whereas the private ones have been spared in these mice because of their low avidity. Thus, according to the TCR affinity model, the latter would be more difficult to tolerize than the former (for a review see reference 41), whereas the emergence of a low-avidity, private response might require additional steps, such as an elevated expression of adhesion molecules or coreceptors, which would favor cross-reactions. The choice of the private repertoire is likely to be influenced by the expansion of various T cell clones during ontogeny and prior experience in adult life. As an alternative possibility, the dominant public responses seen here and in other experimental models (cf. above) could be explained by the existence of a powerful positive selection process involving an as yet unknown selecting element. If private T cell clones may undergo positive selection, it is interesting to consider that the positively selecting elements might themselves include public elements (displayed by all mice) and private ones (of a more random nature), thus yielding public and private T cell clones. The presence of public and private components in T cell responses is reminiscent of the situation observed with antibodies, where responses with recurrent idiotypes are found together with a cortege of nonrecurrent ones (42). Furthermore, when structural genes controlling the expression of recurrent idiotypes in response to various antigens were analyzed, it was found that each family of idiootype-positive antibodies was encoded by a limited number of VL genes and by one VH and JH gene segment (43–46). B lymphocytes bearing such dominant idiotypes disappear after neonatal suppression with antiidiotypic antibodies. In the neonatally suppressed mice, B lymphocytes with alternative idiotypes are taking over in response to the same antigen. Thus, although T and B cell repertoires are selected differently, clonal dominance is observed in both compartments and coexists with private distinct repertoires. Such a finding may have important implications in autoimmune situations where therapeutic attempts to inactivate dominant autoreactive lymphocytes may fail, since new antiself clones may replace the initial autoreactive T cells and exert potentially similar pathogenic effects.

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