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Increased Peptide Promiscuity Provides a Rationale for the Lack of N Regions in the Neonatal T Cell Repertoire

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Summary

Making use of mice deficient for terminal deoxynucleotidyl transferase (TdT) expression and a random peptide library, we have examined the diversity and peptide specificity of the neonatal T cell repertoire specific for a single H–2D^b-restricted peptide. Consistent with the predicted decrease in repertoire diversity, polyclonal CTL lines and individual clones from different TdT° mice are more similar to each other than those from different wild-type mice in terms of their fingerprints of cross-reactivity to the library and their TCR sequences. We have also found that several TdT° CTL clones cross-react with many more library peptides than wild-type CTL clones. In a few instances, the degree of peptide promiscuity correlates with TCR sequence characteristics such as N region addition and homology-directed recombination, but not CDR3 loop length. Based on epitope titrations for each clone, TCR affinity for antigen is consistently high; thus, this reduced specificity for peptide may coincide with an accentuated affinity for the α helices of the MHC. Peptide promiscuity in the neonate may allow the relatively small numbers of T cells in the periphery to protect against a broader range of pathogens.

Introduction

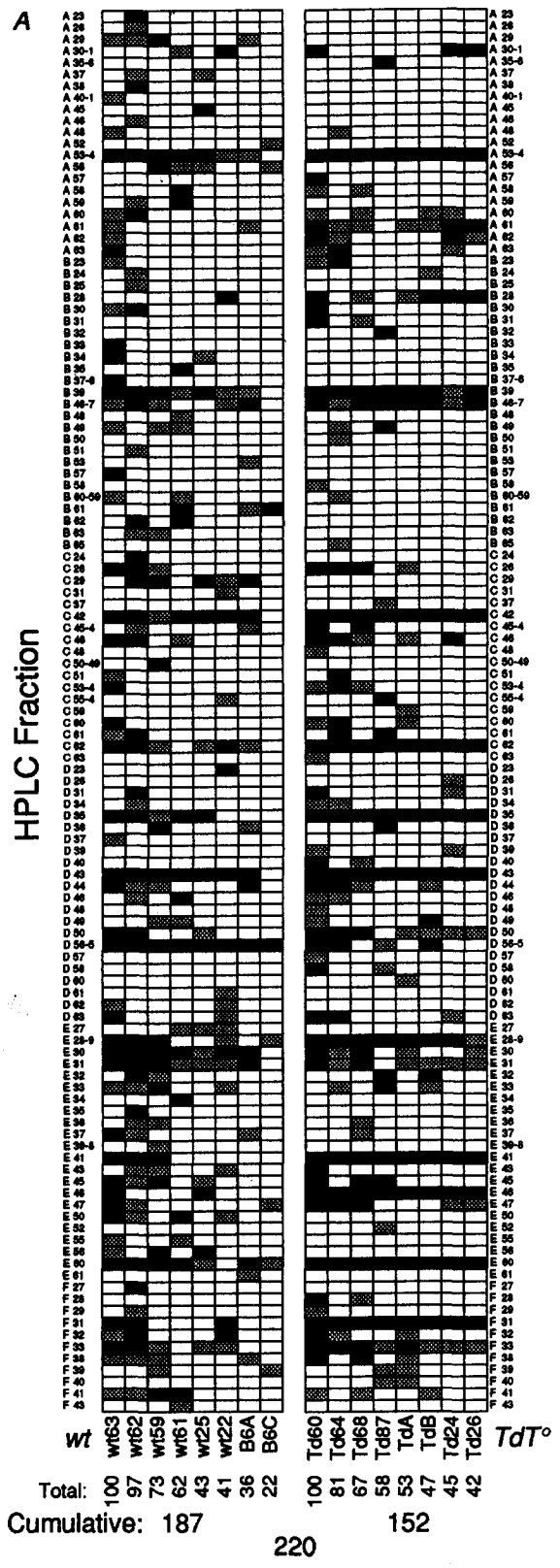
Lymphocytes are capable of responding to a great diversity of antigens. The multiple specificities of their antigen receptors is generated, in part, from the combinatorial usage of large families of gene segments. Both B and T cells produce heterodimeric receptors in which the antigen-binding domain is encoded by variable (V), diversity (D), and joining (J) segments for one chain and V and J segments for the other. Further diversification derives from imprecision in recombination, driven by nucleases that process the gene segment termini and a template-independent polymerase, terminal deoxynucleotidyl transferase (TdT), that randomly inserts nucleotides between receptor gene segments prior to ligation (for review see Lieber, 1991). Based on predicted and solved T cell receptor (TCR) structures, several domains of each chain fold together to form the antigen-binding surface (Bentley et al., 1995). Of these, the complementarity determining region-3 (CDR3) is encoded by the last few nucleotides of the V segment, the J or DJ segments, and the intervening N regions. Thus, the CDR3 possesses much of the heterogeneity. Unlike immunoglobulin, a TCR will only recognize short peptides bound within the groove of major histocompatibility complex (MHC) class I or class II molecules, and a growing body of evidence indicates that the CDR3 interacts more closely with the peptide, while the domains encoded by the V gene segments bind the MHC α helices on either side of the presented peptide (Jorgensen et al., 1993; Hong et al., 1992; Wither et al., 1991; Nalefski et al., 1992). The most diverse portion of the TCR is, therefore, responsible for screening a panoply of self- and foreign peptides presented by an oligarchy of MHC molecules.

In mice, TdT is not expressed in developing thymocytes until 4–5 days after birth, and significant N region addition is not observed in single-positive thymocytes until day 8 (Bogue et al., 1992). Thus, the newborn αβ TCR repertoire is devoid of N regions and, likewise, far less diverse than that of an adult (Bogue et al., 1991; Feeney, 1991). What is the role of this early repertoire? Because the structural effects of TdT expression are observed only in the CDR3, N region addition should modulate the fine specificity of peptide recognition. Making use of a new reagent for measuring the degree of TCR–peptide interactions (Gavin et al., 1994), we have conducted an analysis of the peptide specificity of neonatal-type TCRs. In addition to describing their limited diversity, our results show that these receptors are far more peptide promiscuous than adult-type receptors. A more cross-reactive repertoire would, therefore, permit the few T cells that populate the periphery of young mice to protect against a multitude of pathogens.

Results and Discussion

A Diminished T Cell Repertoire

We have previously observed that the peptide libraries are a useful reagent for visualizing the diversity of an antigen-specific T cell repertoire. The libraries consist of large pools of random peptides with fixed MHC anchor residues (Gavin et al., 1994). Following high pressure liquid chromatography (HPLC) fractionation, the peptides may be used to sensitize ^{51}Cr-labeled target cells for lysis by cytotoxic T lymphocyte (CTL), and the pattern of antigenic fractions delineates the fine specificity of the TCRs. When used to screen the library, three separate polyclonal CTL lines, raised in parallel against the same epitope, gave unique patterns of fine specificity (Gavin et al., 1994). Thus, from three mice, three unique sets of TCRs were elicited, underscoring the fact that a single mouse contains a small subset of the potential T cell repertoire. Would this also be the case with TdT° mice, or would the CD8$^+$ T cell repertoire be sufficiently smaller as to yield similar TCRs from each mouse upon immunization? To address this question, the D^b-restricted peptide library was screened with several wild-type (designated with the prefix B6 or wt) and TdT° CTL lines specific for the nucleoprotein of influenza A/PR/8/34 ($NP_{366–374}$), and their fine specificities were compared. Our results show that while the patterns of cross-reactivity, or fingerprints, of the TdT° lines were not identical to each



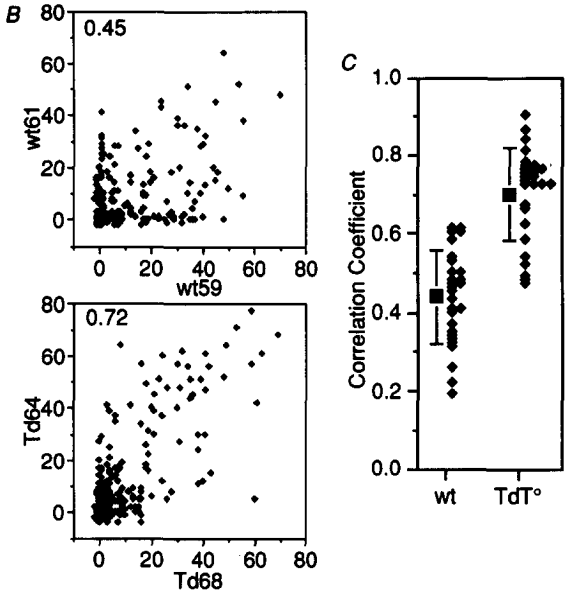


Figure 1. Polyclonal CTL Lines from TdT° Mice Are More Similar to Each Other than Wild-Type Lines

(A) The peptide library, consisting of ten pools (A–J) of 186,000 peptides, each separated into approximately 43 HPLC fractions, was screened with CTL lines specific for influenza A/PR/8/34 $NP_{366-374}$. Each line was raised from an individual mouse and used to screen the library as described (Experimental Procedures). Approximately half of the library is shown, such that the rows represent the HPLC fractions that contained greater than 10% specific lysis for at least one CTL line, and the columns represent individual lines. Closed, dark stippled, and light stippled boxes represent greater than 30%, 20%, and 10% specific lysis, respectively. Consecutive positive fractions that always gave the same peak of activity were considered to represent one peptide and were listed as such with the most active fraction first (D56-5) (see Figure 2A, B6A6). If the two fractions were recognized differently by two CTL lines, they were listed and counted separately (D35, D36). The total number of positives (>10% specific lysis) for the whole library is listed for individual lines, all lines of one genotype, and all lines taken together.

(B) Comparisons of two normal and two TdT° lines. Each data point represents the percent specific lysis values of a single HPLC fraction for both CTL lines. The data were taken from the reduced matrix of lysis values as depicted in (A), except the full data set was used, rather than the half shown in (A). The corresponding correlation coefficients are shown.

(C) The correlation coefficient for each pair of lines is shown, with their averages and standard deviations.

other, they were noticeably more similar. Figure 1A shows the fingerprints for approximately half of the library. When the full data set was analyzed, seven or all eight wild-type lines shared 14 mimotopes in comparison to 31 by seven or all eight TdT° lines (>10% specific lysis). Likewise, 118 peptides were recognized by one or two wild-type lines, while 78 were detected by one or two TdT° lines.

To quantitate the similarity of the fingerprints, the correlation coefficient for each pair of lines of a single genotype was calculated. Figure 1B shows the comparisons of two wild-type and two TdT° lines and their respective correlation coefficients. In such an analysis, randomly distributed data points and a linear array of points yield respective correlation coefficients of 0 and 1. When all pairs of lines

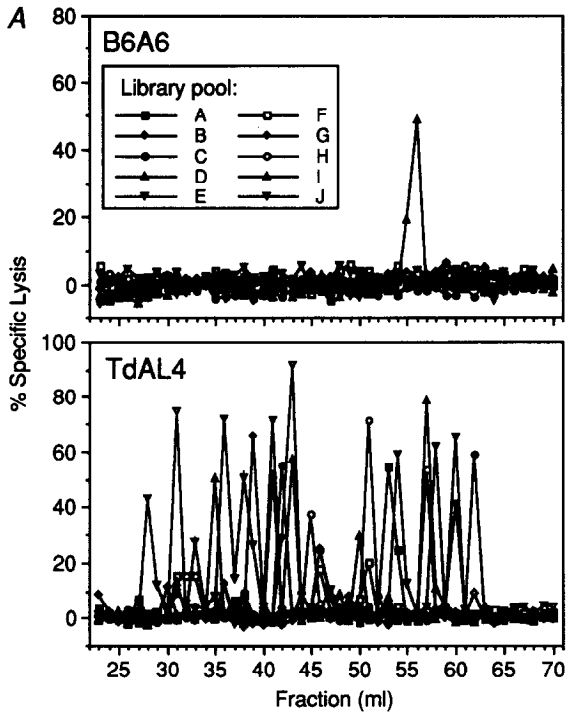
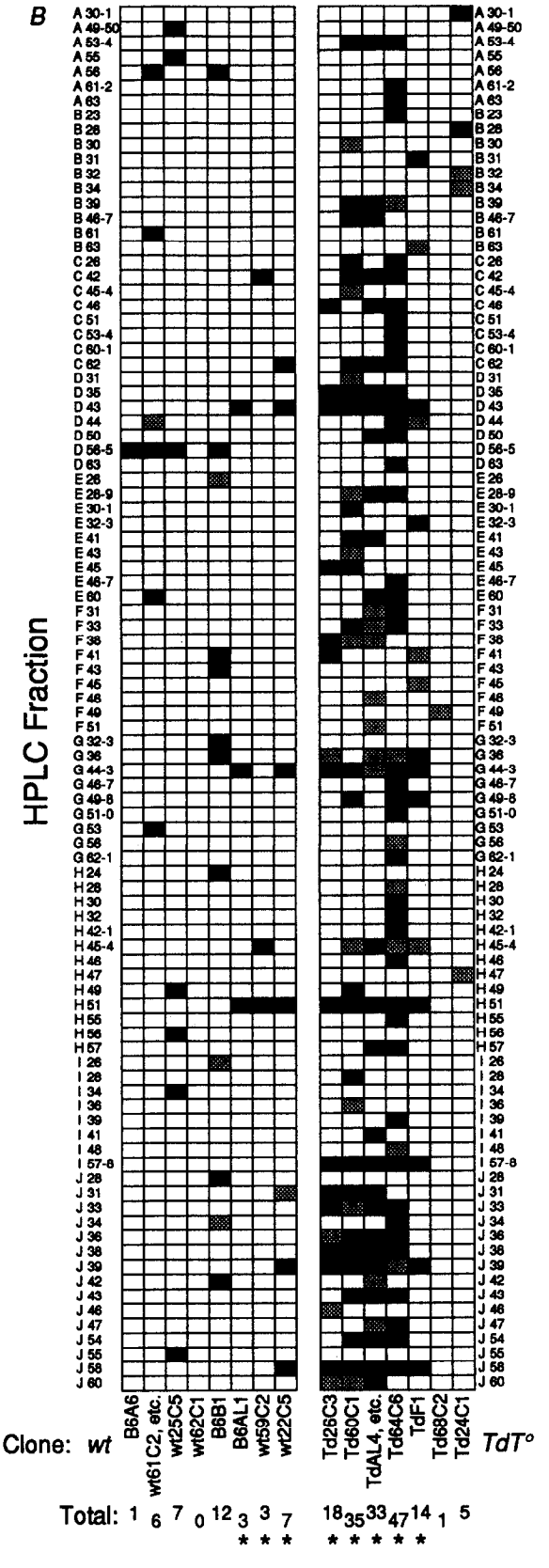


Figure 2. Several TdT° CTL Clones Exhibit Increased Peptide Cross-Reactivity

(A) Peptide specificity fingerprints of a representative clone from each genotype are shown. The ten symbols represent the ten library pools. The fingerprint for TdAL4 was extremely similar to three other CTL clones bearing the same TCR.

(B) The library was screened with a total of 19 CTL clones, each raised from an individual mouse. Positives were tabulated as for Figure 1A. Clones wt61C2 and B6CL3 expressed identical TCRs at the amino acid sequence level, and clones TdAL4, TdC4, TdBL2, and Td87C3 expressed TCRs with the same nucleotide sequences. Clones that utilize Vβ8.3, Jβ2.2, and Vα16 are marked with an asterisk.



were analyzed, the wild-type and TdT° lines gave average correlation coefficients of 0.44 and 0.70, respectively (Figure 1C). Thus, the TdT-independent antigen-specific repertoire is substantially more reproducible among individual mice.

TdT° Clones are Peptide Promiscuous

To look for an effect of TdT expression on the peptide specificity of individual TCRs, the peptide library was screened with several CTL clones from normal and TdT° mice. Figure 2A shows the results for two representative clones. While wild-type clone B6A6 responded to only 1 peptide mimotope in the library, the mutant clone TdAL4 detected 33. Each peptide clone was represented at ~100 pM during the assay. This was confirmed by the observation that the targeting activity decreased at a 1:100 dilution of the positive fractions, consistent with the 1–0.1 pM limit of peptide detection by CTL (data not shown). In all, 19 clones were used to screen the library. Four TdT° clones gave very similar fingerprints and were later found to express the same TCR (see below). The same was true for two wild-type clones; thus, Figure 2B shows the results

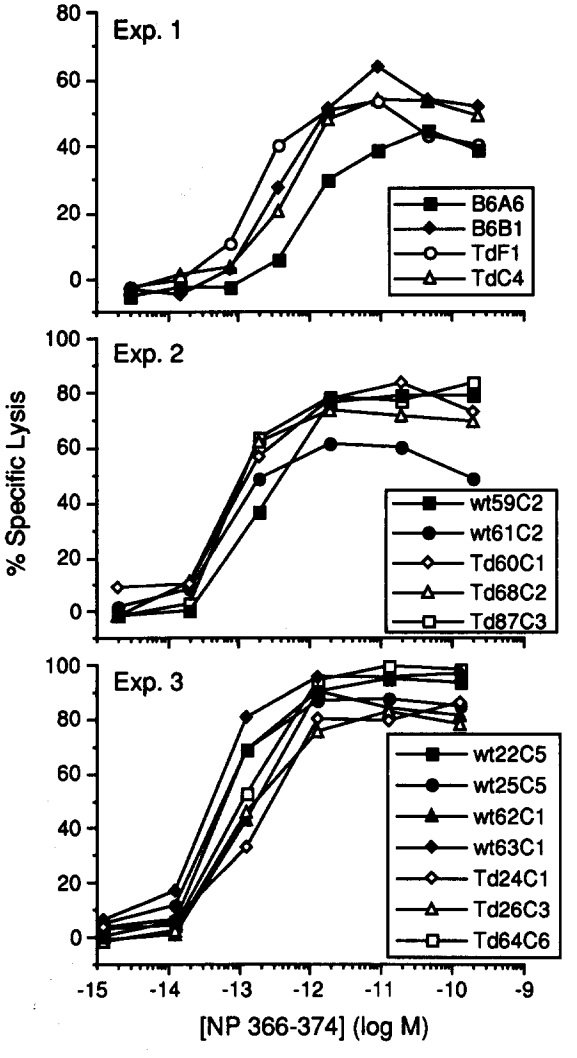


Figure 3. Both Groups of CTL Clones Detect Subpicomolar Levels of their Antigenic Peptide

The influenza A/PR/8/34 $NP_{366-374}$ peptide was titrated for recognition concomitant to the screening of the library. The experiments were performed on different days with similar numbers of TdT^+ (closed symbols) and TdT° (open symbols) clones.

for 15 unique TCRs. Among the wild-type clones, only one (B6B1) detected over 10 peptides, and another (wt62C1) did not react with any peptides. While two TdT° clones also displayed high peptide specificity (Td68C2, Td24C1), the rest were remarkably more cross-reactive, detecting from 14 up to 47 mimotopes.

The low cross-reactivity observed for many of the clones may have resulted from a requirement for higher concentrations of peptide, such that only a few library peptides were represented at detectable concentrations. A titration of the $NP_{366-374}$ epitope suggested that this was not the case. All of the clones detected less than 1 pM of this epitope, and the slight differences in peptide sensitivity that were evident did not correlate with cross-reactivity (Figure 3). In addition, as mentioned above, titration of positive fractions confirmed that the mimotopes were represented at ~100× detection levels.

TCR Sequences of Cross-Reactive CTL

We then sequenced the TCR junctional regions of the clones to look for a correlation between particular structural characteristics and peptide cross-reactivity. The $NP_{366-374}$-specific repertoire has been reported to be dominated by CTL expressing Vβ8.3 (Deckhut et al., 1993). Similarly, 9 of the 15 unique TCRs in the current study utilize Vβ8.3, which is always paired with Vα16, and 8 of these use Jβ2.2. Interestingly, this combination of gene segments seems to confer cross-reactivity to library fraction H51 (see Figure 2B; Figure 4B). All of the highly cross-reactive TdT° clones express these gene segments, and the six clones that utilize other Vβs and Vαs are very peptide specific. Only the most cross-reactive clone, Td64C6, contains two productive TCRα rearrangements. While we cannot exclude the possibility that both α chains contribute to mimotope recognition, it is more probable that only one receptor is restricted to D^b and capable of recognizing foreign peptides in its context. Because Vα16 is always paired with Vβ8.3 among the other clones, it is likely to be the operative α chain for this clone, as well.

The lack of TdT involvement in the neonatal T cell repertoire results in CDR3 loops that are on the average 1 aa shorter than those of the adult (Bogue et al., 1991); therefore, the first structural feature we scrutinized was CDR3 loop length. Longer CDR3s may be forced to make more intimate contact with peptides, thus raising the peptide specificity of the TCR. Among these clones, the CDR3 loops do not differ dramatically in length, and the small differences that exist do not coincide with cross-reactivity (Figure 4B). This is true even for the $V\beta8.3^+V\alpha16^+$ clones. For example, among these nine receptors, the TCRβ CDR3 loop is consistently 9 aa in length and the TCRα CDR3 varies from 8–10 aa. The three clones with the longest CDR3 recognized 3, 7, and 35 mimotopes, and the five clones with the shortest recognized 3, 12, 18, 33, and 47 mimotopes (Figure 4B).

The ablation of TdT was found to increase homology-directed recombination as well as abolish N region addition (Komori et al., 1993; Gilfillan et al., 1993), and a better correlation exists between cross-reactivity and these two structural characteristics. The five cross-reactive TdT° TCRs all share the same β chain. This sequence results from 2 nt of homology-directed recombination at each join. One clone (Td60C1), however, produces the same chain while inserting an N nucleotide at the V–D join (Figure 4). (N regions are not completely absent from the TdT° T cell repertoire [Komori et al., 1993; Gilfillan et al., 1993].) Of these five TCRs, one is expressed by four independently isolated clones (TdAL4, etcetera), and its α chain also results from homology-directed recombination. The Vα16 chain of the most cross-reactive clone (Td64C6) also contains a homologous join, and the two TdT° clones of intermediate cross-reactivity (TdF1 and Td26C3) do not (Figure 4). In addition to its β chain, the α chain of Td60C1 also contains an N nucleotide; however, this clone remains considerably cross-reactive. The genotype of this clone was confirmed by polymerase chain reaction (PCR) to be TdT°. In contrast with the TdT° clones, the wild-type clones expressing Vβ8.3–Jβ2.2 joins substituted the Ala

A **TCRβ sequences** **TCRα sequences**

	V	P	N	P	D	P	N	P	J	V	P	N	P	J	X-R
B6A6	5.1	AGCTCT	G	C	GGACAG		A	AGGAA	TTCCTATAATTCG 1.6	1	G			TGAATTATGGGAGCA	25	1
B6CL3	5.2	AGCTCT	AG		CTGGGGGG		T		T 2.7	3	GTGAG		AT	TAACAGGCA	30	6
wt61C2	5.2	AGCTCT	TC		CTGGGGGG		T		T 2.7	3	GTGAG		GT	TAACAGGCA	30	6
wt25C5	8.1	AGCAGTGAT	C		ACA		AG		AACACA 1.1	5	GTCAGT		CC	C GTTAATACAG	33	7
wt62C1	2	TGCAGTGC	C		CTGG		AGAT		TCAAAAC 2.4	8	TTGAGTGATCG		AAA	AGGAA	34	0
B6B1	8.3	AGCAG	C		GACTGGGG				GAGTCAAAAC 2.4	16	ATGAGA			ACTGCCA	19	12
B6AL1	8.3	AGCAGTG			GGGG		TT		CAAACACCGGG 2.2	16	ATGAGAG			CAAATT	11	3
wt59C2	8.3	AGCAGTG			GGGGG		T		CAAACACCGGG 2.2	16	ATGAGAG		C	TTCTGGAGGAA	34	3
wt22C5	8.3	AGCAGTG	G		TGGGGG				AAACACCGGG 2.2	16	ATGAGA		TC	TTCTGGAGGAA	34	7
Td26C3	8.3	AGCAGTG			GGGGG				GCAAACACCGGG 2.2	16	ATGAGAGAGGGC			T	11	18
Td60C1	8.3	AGCAGTG	G		TGGG				GCAAACACCGGG 2.2	16	ATGAGAG		T	TTCTGGAGGAA	34	35
TdAL4, etc.	8.3	AGCAGTG			GGGGG				GCAAACACCGGG 2.2	16	ATGAGA			ACTGCCA	19	33
Td64C6	8.3	AGCAGTG			GGGGG				GCAAACACCGGG 2.2	16	ATGAGAG			CCAGG	12	47
										34S	GTGGA			CAACA	8	
TdF1	8.3	AGCAGTG			GGGGG				GCAAACACCGGG 2.2	16	ATGAGAGAGG			CAGGCA	BVI	14
Td68C2	5.1	AGCTCTCTC			TGGGGGGGC				GAC 2.5	6	CTGAGAGTAGC	G		GGCA	49	1
Td24C1	7	AGCAG			ACAGGGGG				CTGGAAAT 1.3	2	GCAAGT			ACTG	10	5

B

Clone	Vβ	CDR3	Jβ	Length	Vα	CDR3	Jα	Length	X-R
B6A6	5.1	SADRRNSYNSPL	1.6	12	1	NYGSSGNKL	25	9	1
wt61C2, etc.	5.2	SSWGVEQ	2.7	7	3	RLTGNTGKL	30	9	6
wt25C5	8.1	SDHKNTEV	1.1	8	5	SPVNTGNYKY	33	10	7
wt62C1	2	SALEIQNTL	2.4	9	8	SDRKGSNAKL	34	10	0
B6B1	8.3	SDWGSQNTL	2.4	9	16	RTASLGKL	19	8	12
B6AL1	8.3	SGGSNTGQL	2.2	9	16	RANSGTYQ	11	8	3
wt59C2	8.3	SGGSNTGQL	2.2	9	16	RASGGSNAKL	34	10	3
wt22C5	8.3	SGGGNTGQL	2.2	9	16	RSSGGSNAKL	34	10	7
Td26C3	8.3	SGGANTGQL	2.2	9	16	REGSGTYQ	11	8	18
Td60C1	8.3	SGGANTGQL	2.2	9	16	RVSGGSNAKL	34	10	35
TdAL4, etc.	8.3	SGGANTGQL	2.2	9	16	RTASLGKL	19	8	33
Td64C6	8.3	SGGANTGQL	2.2	9	16	RDQGGRAL	12	8	47
					34S	DNMGYKL	8	7	
TdF1	8.3	SGGANTGQL	2.2	9	16	REAGNTRKL	BVI	9	14
Td68C2	5.1	SLWGGDTQ	2.5	8	6	RVAGTGSKL	49	9	1
Td24C1	7	RQGAGNTL	1.3	8	2	STGGYKV	10	7	5

Figure 4. Nucleotide and Amino Acid Sequences of the TCR Junctional Regions

(A) Sequences were determined by direct sequencing of RT–PCR products or by sequencing a few clones of cloned RT–PCR products. Vβ usage was first determined by FACS. A panel of 19 Vα-specific primers was used in conjunction with a Cα primer to determine Vα usage (Casanova et al., 1991). The nucleotide sequence of clone TdAL4 is identical to that of three other clones (TdC4, TdBL2, and Td87C3), each derived from a separate mouse. The sequences for B6CL3 and wt61C2 encode identical receptors. While two rearranged TCRα loci were not uncommon, only clone Td64C6 contained two in-frame joins. The Jα assignments were based on those of Koop et al. (1992); however, our Jα19 lacked a guanine at its 5′ end and yet was identical to two other reported Jα segments (Iwashiro et al., 1993; Yague et al., 1988). In addition, JαBVI was not found in Koop et al., but was described elsewhere (Heuer et al., 1991). Underlined nucleotides are homologous to the adjacent gene segment. The genotype of each clone was confirmed by PCR. X-R, cross-reactivity or the number of positive HPLC fractions.

(B) The translations of the CDR3 regions are shown.

in the fourth position of the CDR3 (CDR3βP4) for a Ser (B6AL1, wt59C2) or a Gly (wt22C5) by inserting N regions. Interestingly, the most cross-reactive of the wild-type clones, B6B1, utilizes the same α chain as the most abundant cross-reactive TCR (TdAL4, etcetera), which results from a homology-directed join (Figure 4).

Owing to the variability in Jβ and Jα usage, conclusions about the effects of TdT on TCR peptide specificity should not be drawn with too much certainty from the full set Vβ8.3$^+$ clones. However, smaller sets of clones do share all gene segments. Clones wt59C2, wt22C5, and Td60C1 utilize Jα34, and clones B6AL1 and Td26C3, Jα11. The effect of TdT on the α chain cannot be assessed among these clones because none have homologous joins or TdT-dependent N region addition. In all cases, though, the canonical TCRβ sequence of the TdT° clones is disrupted in these wild-type clones by N region addition and a partial loss of homology-directed recombination. It is surprising that the resulting subtle changes in amino acid sequence have such a dramatic effect on cross-reactivity. These substitutions parallel a loss of reactivity to particular mimotopes without the addition of new specificities. Clone wt22C5 differs from Td60C1 by Ala→Gly and Val→Ser mutations in CDR3βP4 and CDR3αP2, respectively, resulting in a loss of recognition of 28 mimotopes. For wt59C2, Ala→Ser and Val→Ala mutations at these positions result in a loss of 32 mimotopes (see Figure 2B). A similar relationship can be observed between clones B6AL1 and Td26C3.

Mimotopes for a TdT$^+$ epitope-specific repertoire cannot always be a subset of those for a TdT° repertoire, however, because our data show that the wild-type CTL lines recognized many more mimotopes than the TdT° lines (see Figure 1A). At the clonal level, most of the mimotopes for the Vβ8.3$^-$Jβ2.2$^-$Vα16$^-$ wild-type clones (see Figure 2B, no asterisk) were not detected by the cross-reactive TdT° clones using these segments. Of these, certain peptide fractions such as A56 and B61 were not detected by any of the polyclonal TdT° lines (see Figure 1A). These data suggest that certain gene segment combinations require

TdT-mediated recombination for the recognition of particular epitopes, and that a more canonical rearrangement of the same segments in the absence of TdT would result in the loss of epitope reactivity. Gilfillan et al. (1995) provide experimental support for this hypothesis, finding that the gene segments of a TCR that dominates the wild-type response to a class II–restricted antigen are rarely used in the TdT° response.

Public and Private T Cell Repertoires

As expected, our data show that the diversity of the TdT° T cell repertoire is substantially reduced, and, more specifically, they describe how the populations of peptide-specific TCRs are distributed among separate mice. A recent study concluded that various TCRs are generated at very different frequencies, such that every animal possesses common (public) and unique (private) T cell specificities (Cibotti et al., 1994). Our results illustrate a TdT° repertoire that is dominated by public specificities, and a wild-type repertoire that is not. In contrast with the diversity of wild-type clones, 4 of our 10 TdT° clones contain the same α and β nucleotide sequences, and 4 others differ only in Jα usage. The fingerprints for these five TCRs are similar to each other; however, the set of mimotopes common to all eight TdT° lines is most similar to the fingerprint of the common TCR, TdAL4 (see Figures 1A and 2B). Thus, the canonical β chain rearrangement is most likely to prevail in each animal in the response to $NP_{366-374}$. The TdAL4 α chain may also be part of the public domain, while the private repertoire would merely consist of other Vα16 rearrangements paired with this public β chain and less frequent TCRs utilizing other Vα and Vβ segments. This dominant repertoire, consisting of merely a single αβ TCR and less frequent usage of alternative Jα segments, is responsible for the high degree of mouse-to-mouse reproducibility depicted in Figure 1C. The TdAL4 fingerprint was not conserved among the wild-type lines, indicating that TdT expression abolishes this specificity. It may, however, be part of the private repertoire, as its fingerprint is present in a few of the most cross-reactive wild-type lines. We do not have evidence for a different wild-type public repertoire. The fingerprint for the TCR shared by clones wt61C2 and B6CL3 is not conserved among the wild-type lines, and some of the mimotopes, such as D56-5, that are antigenic for every wild-type line can be recognized by very dissimilar wild-type TCRs (see Figures 1A and 2B).

TCR-MHC Interactions and the Perinatal Repertoire

We have found that TCRs that are generated in the absence of TdT can have markedly reduced peptide specificity, and that the homology-directed rearrangement of gene segments may contribute significantly to this phenotype. How do peptide-promiscuous and peptide-specific TCRs differ in their association with MHC–peptide complexes? It is likely that they do not differ in terms of overall affinity for MHC + peptide because a titration of $NP_{366-374}$ showed that both types of CTL responded equally well to subpicomolar levels of peptide. We, therefore, propose that a resegregation of affinities between the two surfaces is occurring, such that peptide-promiscuous TCRs make more contacts with the MHC α helices and fewer contacts with the peptide (Figure 5). Another possibility is that the CDR3s of cross-reactive receptors are more flexible, changing conformation to associate with various peptides. There is no correlation, though, between cross-reactivity and the number of CDR3-encoded glycines. Perhaps the amino acid substitutions discussed above, in particular CDR3βP4 Ala→Ser, induce a more rigid conformation to the loops by intramolecular forces.

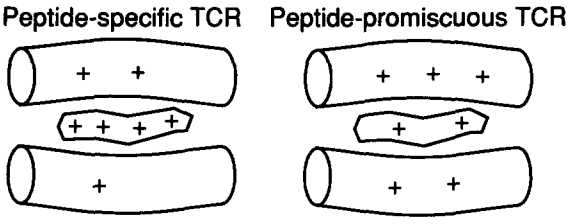


Figure 5. A Model Depicting the Difference in Ligand Binding between Peptide-Specific and Peptide-Promiscuous TCRs, in which Crosses Represent Contact Points and Both TCRs Exhibit the Same Overall Affinity for the Peptide–MHC Complex

In either case, peptide promiscuity is a characteristic unique to the TCR; thus, it should also influence T cell selection events in the thymus. Our results are consistent with the finding that a greater proportion of TdT° thymocytes undergo positive selection (Gilfillan et al., 1994). Recent experimental evidence supports the model that thymocyte selection is dependent on specific interactions with self-peptide ligands (for review see Jameson et al., 1995). TCRs that make more intimate contact with the MHC and less with the peptide are likely to be positively selected by a broader assortment of self-peptides. In generating TCRs with a more stringent requirement for specific peptides, TdT-mediated recombination will disrupt the putative intrinsic affinity TCRs possess for MHC (Blackman et al., 1986), resulting in the neglect and apoptosis of more double-positive thymocytes.

The lack of perinatal TdT expression is advantageous for two reasons. More efficient positive selection results in a rapid export of the early αβ T cell repertoire. In addition, our findings indicate that the few T cells present in the periphery of a 1-week-old mouse could protect against a wide range of diverse pathogens. A mouse of this age contains approximately 10^5 $CD8^+$ T cells (Kelly and Scollay, 1992; unpublished data). However, the precursor frequency of the naive adult $CD8^+$ repertoire specific for a single antigen is less than 10^{-6} (Selin et al., 1994). With a newborn repertoire of more cross-reactive CTL, this frequency might be increased to a level adequate for the size of the animal. The frequency of CTL precursors that cross-react to two unique pathogens (Selin et al., 1994) may also be elevated.

By the same token, with its increased affinity for self-MHC, this repertoire might be more inclined to cause autoimmune disease. While the autoimmunity observed in

mice following neonatal thymectomy may result from a paucity of regulatory CD4+ T cells (Bonomo et al., 1995), there also exists evidence suggesting that the threshold for central tolerance is raised in newborn mice, making thymocytes partially refractory to deletion (Bonomo et al., 1994; Rueff-Juy et al., 1991). The resegregation of receptor contacts that we propose would not require such a modulation to induce increased positive selection; however, the synergy of both events would allow for an even more rapid export of the early αβ T cells. Analysis of mice transgenic for TdT under the control of a more constitutive promotor would help answer the remaining questions about the functionality of this repertoire. The TdT° mice have been challenged with several pathogens and antigens, and no immunodeficiency has been observed (Gilfillan et al., 1995). It is interesting, therefore, to speculate that TCR N region addition has evolved not only to provide an extremely diverse T cell repertoire, but also to lower the risk of pathogen-induced autoimmunity caused by cross-reactive T cells.

Experimental Procedures

Mice

Female 6-week-old C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). TdT° mice were a gift of D. Mathis and C. Benoist (Gilfillan et al., 1993). Genotypes were determined by PCR of tail DNA using a mixture of the following primers, which amplified a 170 bp wild-type fragment and a 210 bp TdT° fragment. TdTa, GATGCCCTTGATATCCTGG; TdTb, CTCTATGATACTCTTCACCTTG; TdTc, ACGCACGGGTGTTGGGTCG.

Cell Lines

Prior to its use as the target cell, the C57BL/6-derived RMA-S cell line (Ljunggren and Kärre, 1985) was cultured over night at 31°C to enhance the number of MHC class I molecules on the surface (RMA–S^{31}). To raise the CTL, mice were immunized intraperitoneally with ~100 HAU influenza A/PR/8/34. After 3 weeks, their splenocytes were stimulated weekly in vitro, the first two times with infected syngeneic splenocytes in RP10 medium (Gavin et al., 1994) and all subsequent times with the influenza A/PR/8/34 nucleoprotein 366–374 peptide, ASNENMETM (Falk et al., 1991a), coated on syngeneic splenocytes in RP10 supplemented with 5% supernatant from concanavalin A-stimulated rat spleen cells. Following 5–8 weeks in vitro, the CTL lines were cloned by limiting dilution. One CTL line and one CTL clone were maintained from each infected mouse and used to screen the library. Lines designated with the prefix B6 were raised from C57BL/6 mice, while those line designated with the prefix wt were raised from $TdT^{+/+}$ littermates of $TdT^{-/-}$ (TdT°) mice. CTL clones were derived from lines with similar names (i.e., clone TdAL4 came from line TdA), and clones B6A6, B6B1, TdC4, and TdF1 came from lines that were not used to screen the library.

Library Screening

The D^b-restricted peptide library (Gavin et al., 1994) consists of 1.86×10^6 peptides of the sequence $XXXXNXXX|_M$, in which X represents 1 of 20 aa and the fixed positions are the anchor residues required for binding to the D^b molecule (Falk et al., 1991b). The peptides are expressed in ten unique pools of Escherichia coli clones as C-terminal fusions to maltose binding protein (Riggs, 1992). Following purification and cleavage from maltose binding protein, the peptides from each pool are separated into 43 fractions by reverse phase–HPLC. For these experiments, 400 ml E. coli cultures of each pool (A–J) yielded ~0.8 mg of peptide. HPLC fractions were resuspended in 1 ml H_2O such that each peptide clone was represented at ~4 nM. Of each HPLC fraction, 5 μl was incubated with 10^4 ^{51}Cr-labeled RMA-S^{31} cells for 30 min at room temperature prior to the addition of CTL, resulting in a concentration of ~100 pM for each peptide. For the polyclonal CTL lines, the assay was always conducted after the fourth in vitro stimulation (i.e., the second stimulation on peptide-coated splenocytes) at an effector:target ratio of ~7:1. CTL clones were added at an effector: target ratio of ~4:1. Plates were incubated for 4.5 hr, after which 100 μl supernatant was harvested and assayed for radioactivity. All data collection and manipulation was fully automated.

TCR Sequencing

RNA was prepared from the CTL clones (RNAzol, Tel-Test, Incorporated, Friendswood, Texas), and cDNA was synthesized from TCR–Cα- (ACGTTCCAGATTCCATG) and TCR–Cβ- (GCCAGCACACGAGGGTA) specific primers according to a standard protocol (GIBCO BRL, Gaithersburg, Maryland). Vβ usage was determined by FACS, and Vα usage was determined by PCR using 19 Vα family-specific primers (Casanova et al., 1991) and a Cα-specific primer (ATACGGATCCCAAAGTCGGTGAACAGGC). TCRβ PCR was performed with the following primers: Vβ2, ACTGATACGGAGCTGAGGCTG; Vβ5, TTGATGACTATCACTCTGA; Vβ7, AAGCGGGAGCATTTCTCCCTG; Vβ8.1, CTCTTCTTTGTGGTTTTGATT; Vβ8.3, ACGCAAGAAGACTTCTTC; and Cβ, TGATGGCTCAAACAAGGA. PCR products were either cloned and sequenced (TA Cloning System, Invitrogen, San Diego, California) or sequenced directly (Dorit et al., 1995).

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