Comparative T cell receptor repertoire selection by antigen after adoptive transfer: A glimpse at an antigen-specific preimmune repertoire

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The low frequency of precursor cells specific for any particular antigen (Ag) makes it difficult to characterize preimmune T cell receptor (TCR) repertoires and to understand repertoire selection during an immune response. We have undertaken a combined adoptive transfer single-cell PCR approach to probe the Ag-specific preimmune repertoires of individual mice. Our strategy was to inject paired irradiated recipient mice with normal spleen cells prepared from individual donors and to compare the TCR repertoires subsequently selected during a CD8 response to a defined model Ag. We found that although some TCRs were shared, the TCR repertoires selected by mice receiving splenocytes from the same donor were not identical in terms of the TCRs selected and their relative frequencies. Our results together with computer simulations imply that individual mice express distinct Ag-specific preimmune TCR repertoires composed of expanded clones and that selection by Ag is a random process.

Effective immune responses against viral pathogens and tumors involve the activation, differentiation, and clonal expansion of T lymphocytes displaying a variety of effector and regulatory functions. Preventative and therapeutic vaccine strategies attempt to optimize these processes. The key components determining the specificity of this selection are the clonally distributed $\alpha\beta$ T cell receptors (TCRs) for antigen (Ag) (reviewed in refs. 1 and 2). The imprecise joining of the large number of variable (V), diversity (D, for β only), and junctional (J) elements within the TCR α- and β-loci, and the addition of extra nucleotides at the joins, contributes to an enormous potential diversity of $\alpha\beta$ TCRs (reviewed in ref. 1). In humans, it has been estimated that individuals express at least 25 million different $\alpha\beta$ TCRs (3). After positive and negative selection in the thymus (reviewed in ref. 4), mature T cells are exported to the periphery and form the preimmune repertoire available for recruitment in immune responses.

TCR repertoires selected by different Ags have been found to vary widely in complexity, and for many responses these are not as diverse as might have been expected from the potential for diversity outlined above (reviewed in refs. 5 and 6). Quantitative comparisons of the TCR repertoires selected by different individuals in response to the same Ag requires the identification of Ag-selected cells directly *ex vivo* and a method of TCR sequence analysis that reflects clonal frequencies. This was possible in the response of DBA/2 mice injected with syngenic (H-2d) P815 cells transfected with the HLA-CW3 gene (P815-CW3 cells) (7–9). These mice develop a strong H-$2K^d$-restricted CD8 response (called the CW3 response) directed predominately against the region 170–179 of the CW3 molecule. CW3-specific cytotoxic T lymphocyte (CTL) clones display a biased TCR repertoire characterized by the apparently exclusive usage of the TCRBV10 (VB10) gene segment, a preferential usage of the TCRBJ1S2 element (JB1.2) and a 6-aa CDR3 region with a canonical SxGxxx motif (10). At the peak of the response, a major proportion of CD8 cells in the circulation and in lymphoid tissues displays a $VB10^+CD62L^-$ phenotype (11), and these can be sorted by flow cytometry as single cells for direct PCR amplification of the rearranged VB10-JB1.2 junctional regions. Our initial single-cell analysis demonstrated that individual mice select distinct and apparently oligoclonal TCR repertoires during the CW3 response (8), and this was confirmed for the response to the homologous HLA-A2 peptide (12, 13). In a recent single-cell PCR analysis, which we carried out to a high level of saturation (9), we found that individual responder mice can select more than 15 different CW3-specific T cell clones. Our study also revealed that the CW3-selected clones were present in a wide range of frequencies and that more than half of the VB10-JB1.2 TCR repertoire was represented by only three clones per animal.

The low frequency of precursor cells specific for any particular Ag makes it difficult to characterize preimmune TCR repertoires. One original approach to this problem was based on the cDNA cloning of VB10-JB1.2 rearrangements size selected to encode 6-aa CDR3 regions typical of TCRs expressed by CD8 T cells specific for CW3 (12). Only a few of the TCR sequences found in such libraries from two mice were identical, which suggested that their preimmune repertoires were not the same. An attempt to follow Ag selection of the repertoire, in which the repertoires in surgically removed half spleens were compared with the CW3-specific repertoires selected in mice immunized after hemisplenectomy (13), suggested that repertoire selection was somewhat random. However, a major limitation to this kind of comparison is that the Ag specificity of the cells expressing the different TCRs identified in the preimmune repertoire is unknown, because their low frequency apparently precluded detection by tetramers (13).

We have undertaken an adoptive transfer (AT) approach as a way to probe the CW3-specific preimmune repertoires present in individual mice. Our strategy was to inject paired irradiated recipient mice with normal spleen cells prepared from single donors and to analyze the CW3-specific TCR repertoires subsequently selected on immunization with P815-CW3 cells. We compare repertoires selected by pairs of mice receiving lymphocytes from the same donor and ask whether they are more likely to share CW3-selected TCRs than those arising from different donors.

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Materials and Methods

Adoptive Transfer and Immunization. Mice were female 8-week-old DBA/2 mice (Iffa Credo). Recipient mice were irradiated with 750 rad ((137)Cs source; CIS Biointernational, Saclay, France) 1 day before AT. Splenocytes were prepared in DMEM (GIBCO/BRL)

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Abbreviations: Ag, antigen; AT, adoptive transfer; TCR, T cell receptor; VB10, TCRBV10; JB1.*n*, TCRBJ1S*n*; CTL, cytotoxic T lymphocyte.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF094483, AF094484, AF094491, AF094496, and AF094500, as well as series AF205719 to AF205777).

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and injected i.p. in a total volume of 0.5 ml DMEM. One day after injection of spleen cells, mice were immunized i.p. with 500 viable P815-CW3 transfectant cells (7) maintained as described (11).

Cell Sorting, PCR Amplification, and Sequencing. Peripheral blood lymphocytes (PBL) were prepared and stained for flow cytometry, and VB10^{+}CD62L^{-}CD8^{+} cells were sorted as single cells for PCR, as described (8, 9, 11). A detailed description of the nested single-cell PCR protocol that amplifies rearrangements of VB10 to JB1.1, 1.2, 1.3, or 1.4, and the sequencing protocol may be found as supplemental material on the PNAS web site (www.pnas.org). Throughout these experiments, a number of precautions were taken to ensure that TCR sequences found to be shared by different mice would not be caused by cellular or molecular contamination. First, mice and cells from mice that had received spleen cells from the same donor or group of donors were not handled consecutively either for cell preparation, flow cytometry and sorting, or PCR amplifications. The fluidic system of the FACStar^{plus} (Becton Dickinson) was extensively rinsed with a bleach solution and then with water between different samples. PCR amplifications were performed on a series of 48 cells at a time (with 12 blanks included in the first PCR plus an additional 6 in the second PCR), and consecutive series were always carried out with cells from different mice, each belonging to a different transfer group.

For convenience, each unique VB10 TCR nucleotide sequence was assigned a three-part code modified from ref. 8. For example, for code 1.1–4c, "1.1" represents the JB gene segment (1S1) used and "4c" identifies the CDR3 region amino acid sequence (1.1–4 = SFGTEV) and nucleotide sequence (1.1–4c = AGC TTC GGG ACA GAA GTC) for that TCRBV10BJ rearrangement.

Computer Simulations. A working version of the simulation program can be found at http://www.isrec.isb-sib.ch/imresim/TCR_selection.html.

Results

Preliminary and control experiments to establish this AT approach for analysis of the preimmune repertoire are described in supplemental material (www.pnas.org).

Two groups of irradiated recipient mice were set up (Fig. 1). In the main group, each mouse within a pair was injected with normal spleen cells prepared from a single donor, and in the second group, each of three mice was injected with cells pooled from the spleens of six donor mice. One day after the transfer, all recipient as well as normal DBA/2 control mice were immunized by injection of 500 P815-CW3 cells. The expansion of VB10^{+}CD62L^{-}CD8^{+} cells, characteristic of the CW3 response, is shown in Fig. 2, and in Tables 5 and 6, published as supplemental data (www.pnas.org).

The single-cell PCR amplification of VB10 TCR rearrangements expressed by CW3-selected (VB10^{+}CD62L^{-}CD8^{+}) cells sorted from PBL from four pairs of single-donor AT mice is summarized in Table 1. Among the eight mice, the proportion of sorted cells amplified by the modified PCR protocol varied from only 2.6% (mouse 13B) to as much as 53.6% (mouse 14A) but was greater than 25% for 6/8 mice. The nonamplified cells most likely represent cells expressing VB10 TCRs rearranged to other JB segments and cells not amplified for technical reasons (PCR efficiency). Inasmuch as similar proportions were amplified in each series of 48 cells from the same mouse (data not shown), these values appear to reflect the relative proportion of each selected VB10 TCR repertoire that is comprised of these 4 BJ rearrangements. Although the proportions amplified were similar for mice from pairs 11A/B (48.3%/41.7%) and12A/B (29.7%/27.1%), this was not the case for the other two matched pairs (Table 1).

Nineteen different VB10 TCR nucleotide sequences were identified in repertoires selected by the single-donor recipient mice

Fig. 1. Adoptive transfer protocol. DBA/2 mice were sublethally irradiated (750 rads) and injected with normal spleen cells 1 day later. In the single-donor group, splenocytes from one donor were transferred to two irradiated recipients; and in the multiple-donor group, splenocytes pooled from six donors were injected into three irradiated recipients. One day after transfer, all mice were immunized with 500 P815-CW3 cells. VB10^{+}CD62L^{-}CD8^{+} cells were sorted as single cells from PBL on day 20–26 after immunization, as indicated in Tables 1–4. Single-cell PCR was performed to characterize the rearranged VB10 TCRs, as detailed in *Materials and Methods.*

(Table 1). Most sequences (15/19) encoded a 6-aa CDR3 region with the SxGxxx motif characteristic of the TCRs of CW3-specific CTL clones (10). One sequence (1.2–73a) encoding a 7-aa CDR3 region with the SxGxxxx motif is similar to those found less frequently in the CW3 response (8, 9). Two sequences encoding the 10-aa CDR3 region VB10 TCRs rearranged to either JB1.2 or JB1.4 were found as multiple copies in mice 14A and 14B, respectively. It is intriguing that VB10-JB1.4 TCRs with this CDR3 length are relatively frequently expressed by CTL clones that recognize an epitope from the homologous region of the HLA-A2/A3/A24 molecules. The remaining atypical sequence encoding an 11-aa CDR3 region was amplified from only one cell. Most of the sequences (11/19) were rearranged to the JB1.2 gene segment, but rearrangements to JB1.1 (3/19), JB1.3 (2/19), and JB1.4 (3/19) were also found in this series of single-donor AT mice.

Six of the nineteen TCR nucleotide sequences identified after single-donor AT were shared by both members of a pair of recipient mice (Table 1). Each of the four pairs shared one TCR rearranged to JB1.2, and two pairs of mice shared an additional TCR rearranged to the JB1.3 gene element. No common TCRs (i.e., identical nucleotide sequences) were found for mice transferred with splenocytes from different donors. In some cases, shared nucleotide sequences were expressed as a similar proportion of the repertoire. For example, TCRs 1.2–9c and 1.2–2m represent 2–3% and 23–27% of the sorted cells of mouse pairs 11A-11B and 12A-12B, respectively. However, the proportion of the repertoire represented by the shared sequences 1.3–2a, 1.2–73a, and 1.2–1h differs by at least 10-fold between members of the same pair.

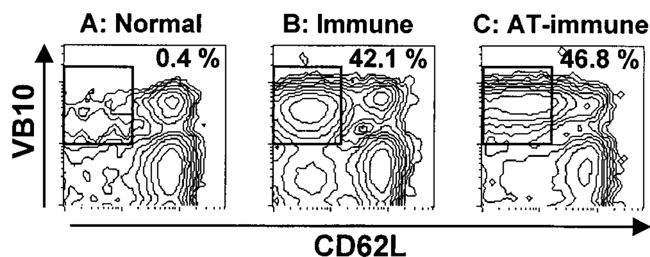

Fig. 2. PBL of AT mice display a phenotype characteristic of the CW3 response. Flow cytometric analysis of PBL from a normal DBA/2 mouse (*A*) or of PBL isolated 3 weeks after immunization of a normal (no. 8, *B*) or an irradiated single-donor recipient (no. 4A, *C*) DBA/2 mouse with 500 P815-CW3 cells. All profiles represent gated $CD8^+$ lymphocytes. The percent of $VB10^+CD62L^-$ cells (region shown) among $CD8^+$ cells is indicated for each profile.

In contrast to the pairs of single-donor AT mice, no shared TCR nucleotide sequences were found among three mice transferred with a pool of spleen cells from six donors (Table 2). Nearly all (13/14) of the TCR sequences encoded a 6-aa CDR3 region with the SxGxxx motif, and all were distinct from those of the single-donor AT mice. Only one copy of a sequence not corresponding to the CW3-specific TCR motif (1.1–24a) was amplified. From two to five different VB10-JB1.2 rearrangements were found for each of the three recipients, as well as at least one additional TCR rearranged to either the JB1.1, JB1.3, or JB1.4 segments.

Our single-cell PCR strategy for repertoire analysis directly reflects cellular frequencies. A comparison of the numbers of cells per clone for the AT mice is presented in Table 3. The sharing of TCR sequences within only matched single-donor AT pairs is also clearly apparent in this table. For 9 of the 11 AT mice, at least one clone of more than 20,000 cells was detected. For three of the four pairs of mice, the shared VB10 TCRs rearranged to JB1.2 represented the largest size clones within the amplified JB classes. Most of the shared clones were present in more than 15,000 copies in at least one mouse of the pair. Many of the clones detected in the three multiple-donor AT mice were of comparable size to those of the single-donor mice for which shared clones were found, suggesting that failure to detect shared clones among these mice was not because of poor clonal expansion.

The CW3-selected TCR repertoires of two normal DBA/2 mice immunized with 500 P815-CW3 cells were also analyzed (Table 4). In our previous studies (8, 9), mice had been immunized with 10–20 million cells. Thirteen or fourteen different VB10 TCR sequences were identified for each of the two mice, and all encoded CDR3 regions with the canonical SxGxxx motif. No common nucleotide sequences were shared by these two control mice. However, three of the sequences, 1.2–3a, 1.1–3a

Table 1. Detection of shared TCRs within each of four matched pairs of mice adoptively transferred with single-donor splenocytes but not among unmatched pairs

TCRBV10 sequence			Repertoire in mouse pairs					
BJ	NS	CDR3	No. of cells	% sort	% BJ	No. of cells	% sort	% BJ
			11A			11B		
1S1	1.1-7a	SRGPEV	56	23.3	100	0	0	0
	1.1-3a	SLGQEV	0	0	0	59	30.7	100
1S2	1.2-11b	SWGSDY	24	10	50	0	0	0
	1.2-3a	SYGSDY	16	6.7	33.3	0	0	0
	1.2-9c	**SQGSDY**	**8**	**3.3**	**16.7**	**4**	**2.1**	**20**
	1.2-3c	SYGSDY	0	0	0	16	8.3	80
1S3	**1.3-2a**	**SRGNTL**	**12**	**5**	**100**	**1**	**0.5**	**100**
	Proportion amplified:		116/240	(48.3%)		80/192	(41.7%)	
			12A			12B		
1S1	1.1-4c	SFGTEV	0	0	0	1	0.5	100
1S2	**1.2-2m**	**SLGSDY**	**44**	**22.9**	**77**	**51**	**26.0**	**100**
	1.2-35a	SSGQDY	13	6.8	23	0	0	0
	Proportion amplified:		57/192	(29.7%)		52/192	(27.1%)	
			13A			13B		
1S2	**1.2-73a**	**SFGASDY**	**64**	**33.3**	**74.4**	**5**	**2.6**	**100**
	1.2-21	SLGSDY	22	11.5	25.6	0	0	0
	Proportion amplified:		86/192	(44.8%)		5/192	(2.6%)	
			14A			14B		
1S2	**1.2-1h**	**SRGSDY**	**96**	**50**	**97**	**7**	**3.6**	**70**
	1.2-76a	SFGTGENSDY	3	1.6	3	0	0	0
	1.2-2c	SLGSDY	0	0	0	3	1.6	30
1S3	**1.3-1b**	**SLGNTL**	**3**	**1.6**	**100**	**1**	**0.5**	**100**
1S4	1.4-11a	RPDREPPNERL	1	0.5	100	0	0	0
	1.4-12a	TGTGFSNERL	0	0	0	10	5.2	90.9
	1.4-9a	SLGQKL	0	0	0	1	0.5	9.1
	Proportion amplified:		103/192	(54.0%)		22/192	(11.5%)	

Single $VB10^+CD62L^-CD8^+$ cells were sorted from PBL of four pairs of single-donor irradiated recipients on day 21 (12A/B and 13A/B) or 26 (pairs 11A/B and 14A/B) after injection of 500 P815-CW3 cells. The TCR VB10 junctional region sequences were determined after a modified single-cell PCR amplification protocol, described in *Materials and Methods,* which amplifies VB10 TCRs rearranged to either the JB1.1, −1.2, −1.3, or −1.4 gene elements. For each sequence, the number (no. cells) and the corresponding percentage (% sort) of positive PCR products of the total number of cells analyzed is shown, as is the percent of the sequence within the JB class (% BJ). The proportion of cells amplified is calculated for the total cells analyzed. Shared sequences are highlighted in bold type.

Table 2. No shared TCRs detected among three mice adoptively transferred with multiple-donor splenocytes

TCRBV10 sequence			Repertoire in multiple-donor recipients								
			21A			21B			21C		
BJ	NS	CDR3	No. of cells	% sort	% BJ	No. of cells	% sort	% BJ	No. of cells	% sort	% BJ
1S1	1.1-6b	SLGQGV	0	0	0	14	7.3	100	0	0	0
	1.1-24a	RMGGLTTKV	1	0.5	100	0	0	0	0	0	0
1S2	1.2-1o	SRGSDY	29	15.1	60.4	0	0	0	0	0	0
	1.2-55a	SLGHLY	9	4.7	18.8	0	0	0	0	0	0
	1.2-1n	SRGSDY	6	3.1	12.5	0	0	0	0	0	0
	1.2-14c	SSGPDY	3	1.6	6.3	0	0	0	0	0	0
	1.2-2i	SLGSDY	1	0.5	2.1	0	0	0	0	0	0
	1.2-42e	SLGPDY	0	0	0	45	23.4	51.7	0	0	0
	1.2-22b	STGTDY	0	0	0	24	12.5	27.6	0	0	0
	1.2-58a	SAGTNY	0	0	0	18	9.4	20.7	0	0	0
	1.2-64a	SLGQSY	0	0	0	0	0	0	64	33.3	98.5
	1.2-1i	SRGSDY	0	0	0	0	0	0	1	0.5	0.5
1S3	1.3-3a	SFGNTL	7	3.6	100	0	0	0	0	0	0
1S4	1.4-8a	SSGERL	0	0	0	0	0	0	21	10.9	100
	Proportion amplified:		55/192			101/192			86/192		

The TCR VB10 junctional region sequences expressed by single VB10$^+$CD62L$^-$CD8$^+$ cells sorted from PBL of three multiple-donor irradiated recipients on day 20 after injection of 500 P815-CW3 cells were determined by the same protocol described for Table 1.

and 1.2–14c, found in immune mouse no. 8, were also found in AT mice 11A, 11B, and 21A, respectively. These were the only sequences found to be shared among mice not belonging to single-donor recipient pairs in these experiments. Their common occurrence is unlikely to be caused by contamination, because immune mouse no. 8 and AT mice 11A, 11B, and 21A were handled in different immunization and sorting experiments, and cells from these mice were never amplified in consecutive PCR experiments. We have previously found identical VB10 TCR sequences (including 1.2–3a) expressed by different CTL clones whose independence could be confirmed by their expression of different TCR α-locus rearrangements. Moreover, the VB10 sequences 1.2–3a and 1.1–3a have been found in other CW3-immune mice analyzed by single-cell PCR (8, 9), and sequence 1.2–3a was also found in cDNA libraries prepared from VB10$^+$CD8$^+$ cells of normal DBA/2 mice (13).

Discussion

Various AT approaches have contributed to our understanding of the different stages involved in B-cell responses to Ag (14–16), and more recently, the AT of T cells from TCR transgenic mice has been used to follow the activation and differentiation of Ag-specific T cells in various immune responses (reviewed in ref. 17). However, to our knowledge this is the first application of AT as a way to probe the Ag-specific preimmune repertoires of normal individual mice. A major goal of the present study was to assess the potential selectability of nearly identical preimmune TCR repertoires *in vivo*. Our results show that, whereas some shared clones are selected in matched single-donor AT pairs, the selected repertoires are not the same in terms of the sequences selected and their relative frequencies.

The AT mice appear to undergo a CW3 response similar to that of normal DBA/2 mice inasmuch as a high proportion (22–60%) of CD8 cells in PBL displayed the VB10$^+$CD62L$^-$ phenotype characteristic of CW3-specific cells. Of the PCR products amplified from a total of 2,592 single cells from the 13 different AT mice in this study, nearly all (839/854, 98%) corresponded to VB10 rearrangements encoding a 6-aa or 7-aa CDR3 region with the SxG... motif typical of CW3-specific TCRs found previously in normal DBA/2 mice immunized with P815-CW3 cells (8, 9). This corresponds to 35 of 39 TCR sequences identified [(Tables 1, 2, and supplemental Table 7) (www.pnas.org)] and implies that most of the VB10$^+$CD62L$^-$CD8$^+$ cells expanded during the CW3 response in these AT mice are indeed CW3 specific. Two of the four noncanonical TCRs, 1.2–76a and 1.4–12a, were found more than once (Table 1) in AT mice 14A and 14B, respectively. Both encode 10-aa CDR3 regions, but only the first (1.2–76a) also encodes the SxG motif. The Ag specificity of cells expressing these TCRs could not be assessed in these experiments, but it is possible that under these conditions, where few clones are selected, rare cells with lower affinity TCRs may be detected in a manner analogous to that of the less "canonical" TCRs found early in the response to cytochrome C (18). Along these lines, it is intriguing that VB10-JB1.4 TCRs encoding a 10-aa CDR3 region with the SxG motif are commonly found among CD8 T cells specific for an epitope from HLA-A2/A3/A24 molecules homologous to that of HLA-CW3 (19). For the purposes of the present study, it is important that the majority of the TCRs amplified from CW3-selected cells in the AT mice closely resemble those found in normal mice directly immunized with P815-CW3 cells.

The AT mice in these experiments appear to select fewer CW3-specific clones than do the directly immunized normal DBA/2 control mice. This may account for the delayed kinetics observed for the expansion of VB10$^+$CD62L$^-$CD8$^+$ cells in the AT animals (supplemental Tables 5 and 6 and data not shown). It will be interesting to test whether induction of the CW3 response by other nonlethal immunogens might allow a more rapid response and possibly the selection of a broader repertoire. However, the priority for this first AT study was to establish conditions under which the selection of the transferred repertoires could be compared, and we believe the pattern of shared clones within pairs of single-donor AT mice validates our approach.

All of the five pairs of single-donor AT mice we analyzed were found to share at least one VB10 TCR nucleotide sequence among the sorted CW3-selected cells. This includes AT pairs 12A-12B and 13A-13B, for which only one or two TCRs were detected in the individual mice; this implies that the selection of only a few clones is not simply because of an inefficiency of the AT protocol. Although we have not formally proven that the VB10 TCRs shared by single-donor AT pairs are associated with the same TCR α-chains, their typical SxGxxx motif and their selection during the CW3 response in matched AT recipients

Table 3. Relative expansion of clones selected during the CW3 response in single-donor and multiple-donor AT mice

TCR BJ	NS	Number of cells per clone in PBL of recipient: 11A	11B	12A	12B	13A	13B	14A	14B	21A	21B	21C
1S1	1.1-3a		47,000									
	1.1-7a	112,000										
	1.1-6b										14,900	
	1.1-4c				800							
	1.1-24a									1,000		
1S2	1.2-1h							99,000	4,200			
	1.2-2m			106,000	39,000							
	1.2-73a					21,000	1,900					
	1.2-64a											22,000
	1.2-42e										48,000	
	1.2-1o									28,000		
	1.2-22b										26,000	
	1.2-2l					7,300						
	1.2-11b	48,000										
	1.2-58a										19,000	
	1.2-3c		13,000									
	1.2-35a			31,000								
	1.2-3a	32,000										
	1.2-55a									8,600		
	1.2-9c	16,000	3,200									
	1.2-1n									5,700		
	1.2-2c								1,800			
	1.2-76a							3,100				
	1.2-14c									2,900		
	1.2-2i									1,000		
	1.2-1i											300
1S3	1.3-2a	24,000	800									
	1.3-3a									6,700		
	1.3-1b							3,100	600			
1S4	1.4-8a											7,100
	1.4-12a								6,000			
	1.4-9a								600			
	1.4-11a							1,000				
Total # of VB10$^+$CD62L$^-$CD8$^+$ cells in PBL:		480,000	150,000	460,000	150,000	60,000	70,000	200,000	120,000	180,000	200,000	60,000

For each clone expressing the indicated VB10 TCR, the number of cells circulating in the blood was calculated from the number of lymphocytes recovered by cardiac puncture, the phenotypic analysis of the PBL, and the proportion of sorted VB10$^+$CD62L$^-$CD8$^+$ cells that express the TCR [Tables 1 and 2; supplemental Table 6 (www.pnas.org), and data not shown].

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makes it highly probable not only that they are CW3 specific but also that they are clonally related T cells.

The extent to which T cells undergo cell division during their normal development in the thymus once they express a functional cell-surface $\alpha\beta$ TCR has not been precisely determined but has been estimated to be at a very low level compared with those at the more immature stages of development (20). Cell division by TCR-expressing CD4$^+$8$^-$ or CD4$^-$8$^+$ thymocytes is apparently more extensive in early postnatal mice than in adults (21). However, many clonally related progeny may fail to reach the periphery, because only a low proportion of thymocytes completes the stages of maturation and positive selection prerequisite for exit from the thymus (22, 23). Mature naive T cells in the periphery appear to be nondividing (24). However, cell division has not been followed for a cohort of normal naive T cells with a defined $\alpha\beta$ TCR in normal mice. The selection of sister clones by AT pairs in our experiments demonstrates that at least some T cells in the peripheral CW3-specific preimmune repertoire exist in multiple copies before selection by Ag.

The patterns of repertoires selected by the single-donor pairs of AT mice in our experiments imply that the CW3-specific preimmune repertoires available in the different donor mice were clearly distinct. First, all of the five pairs of AT mice share at least one VB10 TCR nucleotide sequence, even under conditions in which only a few clones were identified. Second, the TCRs shared (at the nucleotide level) by matched pairs of mice were not found in the other pairs, and each pair selected distinct TCRs compared with the other pairs. If the selectable preimmune repertoires of the different donor mice had been identical, shared TCR nucleotide sequences should have been found as frequently between mice transferred with cells from different donors as between mice belonging to matched pairs. Indeed, shared TCR nucleotide sequences would have been expected at a similar frequency for the multiple-donor AT group if the preimmune repertoires of the six donors had been identical.

As detailed in the supplemental data (www.pnas.org), our AT data can be combined with computer simulations (http://www.isrec.isb-sib.ch/imresim/TCR_selection.html) to test various models for the composition of the CW3-specific VB10 TCR repertoire. Intuitively, if there were too many clones available for selection, it is unlikely that we would have found shared clones for all of the matched single-donor pairs of AT mice analyzed under conditions in which very few clones are detected for each recipient. Our single-cell PCR procedure most directly reflects the relative sizes of the clones expanded during the CW3 response, and these were found to be quite variable within individuals. This characteristic allowed us to reject models of uniform modes of selection that predict little variation in detectable clone sizes. During the CW3 response, fewer than 10 cells from the transferred repertoires would

Table 4. Distinct TCR repertoires selected in two control DBA/2 mice immunized with 500 P815-CW3 cells

Control mouse	TCRBV10 sequence			Repertoire		
	BJ	NS	CDR3	No. of cells	% sort	% BJ
No. 32	1S1	1.1-12a	SFGTKV	24	12.5	85.7
		1.1-11b	SYGEEV	2	1.0	7.1
		1.1-3c	SLGQEV	1	0.5	3.6
		1.1-22a	SRGQEV	1	0.5	3.6
	1S2	1.2-6a	SFGSDY	23	12.0	62.2
		1.2-66a	SVGDYY	10	5.2	27.0
		1.2-1p	SRGSDY	1	0.5	2.7
		1.2-2o	SLGSDY	1	0.5	2.7
		1.2-65a	SAGSDY	1	0.5	2.7
		1.2-74a	SLGHYY	1	0.5	2.7
	1S4	1.4-1a	SFGERL	24	12.5	92.3
		1.4-7a	SSGTGL		0.5	3.8
		1.4-3c	SYGERL	1	0.5	3.8
		Proportion amplified:		91/192	(47.4%)	
No. 8	1S1	1.1-3a	SLGQEV	22	11.5	55.0
		1.1-2a	SLGTEV	14	7.3	35.0
		1.1-4a	SFGTEV	2	1.0	5.0
		1.1-2f	SLGTEV	1	0.5	2.5
		1.1-23a	SLGERV	1	0.5	2.5
	1S2	1.2-42b	SLGPDY	10	5.2	35.7
		1.2-3a	SYGSDY	8	4.2	28.6
		1.2-42f	SLGPDY	4	2.1	14.3
		1.2-1m	SRGSDY	2	1.0	7.1
		1.2-14c	SSGPDY	2	1.0	7.1
		1.2-2n	SLGSDY	1	0.5	3.6
		1.2-39b	SYGPDY	1	0.5	3.6
	1S3	1.3-1a	SLGNTL	8	4.2	100
	1S4	1.4-4a	SLGERL	6	3.1	100
		Proportion amplified:		82/192	(42.7%)	

The TCR VB10 junctional region sequences expressed by single VB10$^+$CD62L$^-$CD8$^+$ cells sorted from PBL of DBA/2 mice on day 20 (no. 32) or day 22 (no. 8) after injection of 500 P815-CW3 cells were determined by the same protocol described for Table 1.

undergo expansion in each recipient mouse, and the expansion would be operationally nonuniform, to give variable-size expanded clones similar to those we detect by PCR. Our simulations attempt to mimic the selection of repertoires similar to those detected at the time of sorting, and they are not meant to describe the intermediate steps involved *in vivo*. For example, the nonuniform mode of selection could arise either from a kinetics effect in which not all cells are triggered simultaneously or from differences in clonal expansion, e.g., because of TCR affinity. Our model of the repertoire would also allow the selection of a larger number of clones with a wide range of clone sizes, as we have described (9) on direct immunization of DBA/2 mice, in that these conditions may allow a greater number of selection events. Moreover, the number of clones selected could also vary with the number of cells injected, as this may alter the number of selection events.

We have described herein an original AT approach for analyzing a preimmune TCR repertoire specific for a defined Ag. Our results directly demonstrate that different mice express distinct CW3-specific preimmune repertoires in which some clones have expanded before immunization. Moreover, these results combined with simulation experiments place limits on the composition of the available preimmune repertoire and on its mode of selection during an immune response. This approach could be applied to other Ag systems and may be useful for understanding repertoire selection under different immunization conditions and vaccine protocols. Our simulation program may by useful for the rational design of new experiments to probe Ag-specific preimmune repertoires by predicting conditions that would clearly discriminate different models.

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