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Facing Two T Cell Epitopes: A Degree of Randomness in the Primary Response Is Lost Upon Secondary Immunization

Philippe Bousso,2* Fabrice Lemaître,* Janine Bilsborough, † and Philippe Kourilsky*

We have analyzed the hierarchy of epitope-specific T cell populations during a primary and a secondary CD8 T cell response. MHC-peptide tetramers were used to track the in vivo kinetics of expansion of T cell populations specific for two Kd-restricted epitopes simultaneously presented by a murine tumor cell following primary or recall immunizations. Individual syngeneic mice generated remarkably different primary CTL responses, as reflected by up to 60-fold differences in the relative contribution of each peptide-specific T cell population to the overall response. In these primary immunizations, the CTL dominance was not dictated by the respective abundance of the presented epitopes. In sharp contrast, the secondary response was systematically associated with a selective expansion of the same epitope-specific population both in vitro and in vivo. In vitro experiments indicated that the extent of expansion of each epitope-specific memory population is modulated by the epitope density. We conclude that, at least for this set of epitopes, the CTL hierarchy is not controlled by the same parameters in a primary vs a secondary response. The Journal of Immunology, 2000, 165: 760–767.

A striking feature of CTL responses is their extreme focusing at the level of epitopes recognized. Even when thousands of potential antigenic determinants are available, very few of them elicit a significant T cell response. Several groups have analyzed the factors that determine the relative magnitude of each epitope-specific T cell population (reviewed in Refs. 1 and 2). Most of these parameters relate to the visibility of the epitope to the immune system depending upon the amount of Ag expressed, the ability of the peptide to be generated in the cytosol, the translocation of the peptide in the endoplasmic reticulum, and the binding of the peptide to MHC class I molecules. In addition, in several instances the availability of the naive repertoire was shown to influence the hierarchy of elicited peptide-specific populations (3–6).

To date, most of these studies were concentrated on primary responses. It remains unclear whether the same parameters dictate the CTL dominance during a secondary antigenic challenge. Primary and secondary responses show major differences. In particular, the latter are more effective and rapid than the former. This could be accounted for by 1) the increased frequency of specific T cells and 2) qualitative changes in the memory cells, which respond more efficiently than naive T cells (7–16). It is therefore possible that distinct mechanisms drive the emergence of dominant CTL populations during an initial or a recall immunization.

Accordingly, we have quantified in individual immune mice the T lymphocytes specific for two Kd-restricted epitopes presented by a transfected tumor cell. Cellular dynamics of peptide-specific T cell populations in vivo were tracked using MHC-peptide tetramers. In addition, through various approaches, we have analyzed the parameters regulating the relative size of each peptide-specific population during the successive phases of an immune response (primary, memory, secondary). CTL dominance in the primary response remarkably differed from mouse to mouse, and extensive variations were observed (up to a 60-fold difference) in the relative magnitudes of the two peptide-specific populations analyzed. We further demonstrated that in a given mouse the primary CTL hierarchy was not dictated by the relative densities of epitopes present. In sharp contrast, a selective expansion of the same peptide-specific population was systematically observed during secondary responses both in vitro and in vivo. In addition, the size of each peptide-specific population during the secondary response was found to be modulated by the relative density of Ag at the surface of the tumor cell. Therefore, the regulation of CTL populations can involve mechanisms of completely different nature during a primary or a secondary T cell response.

Materials and Methods

Mice, cell lines, and chemicals

Female DBA2 (Ly 5.2) mice were purchased from IFFA-Credo (l’Abresle, France). DBA/2-Ly 5.1 have been previously described (17) and were bred in our animal facility. 9.4 T cell hybridoma as well as P815 transfectants have been described previously (18–20). Immunizations were performed by i.p. injection of 10⁷ tumor cells. Adoptive transfers were performed by i.v. injection of the indicated number of naive splenocytes. The CW3 peptide corresponding in sequence to aa 170–179 of the HLA-CW3 molecule (RYLKNGKETL) and the HA peptide (YTVQASSVL) were purchased from NeoSystem (Strasbourg, France). The P1E peptide (GYCGL-RGTGV) and the anti-P1E CTL clone 89/62 were obtained as previously described (21).

In vitro restimulation of memory cells

Splenocytes from immune mice (4 mo after primary immunization) were labeled with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE); Molecular Probes, Eugene, OR. Cells were...
resuspended to 5 x 10^6 cells/ml in PBS with 1 μM CFSE and were incubated for 15 min at 37°C. Cells were extensively washed and placed in a 24-well plate at a concentration of 4 x 10^5 spleen cells/well in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 1-glutamine, β-ME, penicillin, streptomycin, and 5% IL-2-producing EL-4 supernatant (EL-4 stimulated with PMA). Stimulator cells were either unpulsed or peptide-pulsed irradiated tumor cells and were used at a concentration of 10^7 cells/well. Peptide-pulsed tumors were obtained by 1-h incubation at 37°C in the presence of 100 μM peptide followed by extensive washes.

**Flow cytometry**

PE-labeled K^d^-CW3 and K^d^-P1E tetramers were prepared as previously described (22). FITC-conjugated anti-CD8 mAb was purchased from Caltag (South San Francisco, CA). APC-conjugated anti-CD8 was obtained from PharMingen (San Diego, CA), and anti-Ly-5.1 mAb was a gift from Dr. A. Cumano (Pasteur Institute, Paris, France). Cells were incubated 1 h with K^d^-peptide tetramers at 4°C, extensively washed, and incubated with the indicated Abs. Flow cytometry was performed on a FACScan (Becton Dickinson, San Jose, CA). A minimum of 2000 events falling in the CD8^+ population was acquired to determine the percentage of peptide-specific T cells within the CD8^+ population.

**Intracellular IFN-γ staining**

Spleen cells were cultured for 6 h in complete medium supplemented with 5% of IL-2-producing EL-4 supernatant and 1 μg/ml Brefeldin A (Golgi-Plug, PharMingen, San Diego, CA) either with or without peptides. Peptides were used at a concentration of 1 μM. The K^d^-binding peptide HA was used as a control. After 6 h of culture, cells were washed and incubated with APC-conjugated anti-CD8 Ab (PharMingen). Cells were then subjected to intracellular cytokine staining using the Cytotip/Cytoperm kit according to the manufacturer’s instructions (PharMingen) and stained with FITC-conjugated anti-IFN-γ (PharMingen).

**TCR sequencing**

Total RNA from PBLs of immune DBA/2 mice was prepared using the TRIZol reagent (Life Technologies). cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase reagent (Life Technologies) and amplified using primers specific for BV10 and BJ1.2 segments or primers specific for BV1 and BJ2.5 segments. PCR products were cloned using the Topo TA cloning kit (Invitrogen, Carlsbad, CA). Sequences were obtained as previously described (17).

**Results**

**Detection of CW3- and P1E-specific T cells**

We have previously described the generation of K^d^-CW3 tetramers and used these reagents to monitor CW3-specific T cells in response to the transfected mastocytoma P815-HLA-CW3 (17, 22). Recently, a major tumor-specific Ag from P815 has been characterized. The P815E antigenic peptide (referred to as P1E peptide) results from a point mutation within a ubiquitously expressed gene and corresponds to a decapetide presented by K^d^ (21). To explore the CTL response against this epitope, we also generated K^d^-P1E tetrameric complexes. The specificity of both K^d^-CW3 and K^d^-P1E tetramers was checked by staining the CW3-specific 9.4 T cell hybridoma and a P1E-specific T cell clone (Fig. 1C). The peak of the response was reached on day 12, at which point a large fraction (1–20%) of the CD8^+ lymphocytes were specific for each of these epitopes. In addition, the CTL response was largely focused on these two epitopes, because 70–80% of the activated cells (CD8^+CD62L low) were either CW3- or P1E-specific (data not shown). Fig. 1D summarizes the magnitude of each peptide-specific response in individual DBA/2 mice. In most animals (12 of 15), the P1E-specific T cell response was dominant. Some mice, however, displayed an inverse hierarchy with a majority of CW3-specific T cells. Interestingly, extensive variations (up to 60-fold differences) were observed when comparing the ratio of anti-P1E to anti-CW3 responses displayed by individual immune mice (Fig. 1D).

**Role of epitope density and T cell repertoire in ruling the primary CTL hierarchy**

To rule out the possibility that the observed variations of CTL hierarchy were due to different courses of antigenic presentation and possibility to different amounts of Ag present, we followed the responses of two distinguishable naive CD8 T cell repertoires in the same mouse. For that purpose, 1 x 10^7 naive splenocytes from a Ly 5.1 DBA/2 were transferred into naive Ly 5.2 DBA/2 mice. Recipients were then immunized with P815 mCW3-B7, and the CTL hierarchy was determined among the Ly 5.1 and Ly 5.2 T cell populations by tetramer staining. This approach offered the opportunity to follow the fate of two T cell repertoires facing the same antigenic stimulation with regard to overall Ag amounts, hierarchy of epitope densities, and duration of epitope presentation. CTL responses were observed in both Ly 5.1 and Ly 5.2 populations, indicating that each of these populations contained a significant
number of CTL precursors before immunization. We reasoned that in the presence of an epitope-specific regulation of the response, Ly 5.1 and Ly 5.2 T cell repertoires should adopt the same hierarchy in a given immune mouse. This was not the case, and as shown in Fig. 2, CTL hierarchies within Ly 5.1 and Ly 5.2 T cells were clearly independent. Thus, in our system, the relative magnitudes of anti-CW3 and anti-P1E CTLs are not controlled at the Ag level during the primary response.

The CTL hierarchy in an individual animal could reflect the relative frequencies of CTL precursors present before immunization. To assess whether the orientation of the immune response was intrinsically determined by the composition of the naive repertoire, a large pool of naive splenocytes (Ly 5.1) was divided into fractions of $2 \times 10^8$ cells that were adaptively transferred into six naive Ly 5.2 DBA/2 mice. Because fractions consisted of a high number of T lymphocytes derived from a homogeneous pool, they should contain approximately the same number of naive specific T lymphocytes. On the same day, recipients were immunized with P815 mCW3-B7, and the response of cells from donor origin was followed in each recipient. At the peak of the response, PBLs were triple stained with K$^d$-peptide tetramers and mAb against CD8 and Ly 5.1. Surprisingly, the CTL hierarchy among cells derived from the same T cell pool but transferred into different recipients was highly variable (Fig. 3), and even completely asymmetrical. Thus, these results support the idea that CTL dominance in this anti-tumor response is not purely determined by the relative frequencies of specific CTL precursors before immunization. Rather, stochastic processes contribute to the individual variability in the primary CTL hierarchy.

**Clonal composition of peptide-specific T cell populations**

To gain insight into the establishment of the primary CTL hierarchy, we analyzed the clonal composition of CW3- and P1E-specific T cell populations. We took advantage of the observation that both P1E- and CW3-specific T cells display a restricted usage of TCR$\beta$-chains. T cells specific for CW3 display an exclusive usage of the BV10 segment, a preferential usage of BJ1.2, a 6-aa long SQXNQDTQ motif (25), and a SXG motif within the CDR3 (24). The response against P1E is mostly composed of clones displaying the BV1-J2.5 combination together with a 9-aa long CDR3$\beta$ containing a SQXNQDTQ motif (25, 26). We prepared cDNA from CD8$^+$-enriched splenocytes of two immune DBA/2 (day 12 postimmunization), cloned BV10-BJ1.2 and BV1-BJ2.5 PCR products and sequenced individual colonies. Sorting peptide-specific T cells with tetramers is not necessary at this point because virtually all BV10-BJ1.2 (respectively, BV1-BJ2.5) rearrangements correspond to CW3-specific (respectively, P1E-specific) T cells in immune animals (P. Bousso, unpublished results). For both CW3- and P1E-specific responses, several distinct nucleotide TCR$\beta$ sequences were identified in each immune responder, indicating that several distinct T cell clones have been recruited during the response. The nucleotide and deduced amino acid sequences obtained in one immune mouse are listed in Table I together with the sequence occurrence. Note that all
obtained CDR3β sequences displayed the expected length and contained the conserved motifs, confirming that they correspond to peptide-specific T cells. Most importantly, the contribution of each clonotype to the response was highly variable (Table I) as we have previously observed in a related antigenic system (17). In each analyzed mouse and for each epitope, one or two clonotypes were found to contribute to the majority of the response. In contrast, other T cell clones were recruited, but only accounted for a minor part of the response. From this analysis, we conclude that the relative size of each epitope-specific T cell pool is not dictated by the number of distinct recruited T cell clones, but, rather, depends mostly on the extent of expansion of one or two dominant clones.

Evolution of the CTL hierarchy during secondary responses

After the primary response against P815 mCW3-B7, a stable pool of epitope-specific memory T cells persisted for months (Fig. 4). Quantification of epitope-specific T cells was performed using either Kd-peptide tetramers (Fig. 4A) or intracellular IFN-γ staining (Fig. 4B). Both assays yielded similar results, indicating that PIEm and CW3-specific memory cells have the ability to respond very rapidly upon a short restimulation with peptide. Next, we analyzed the evolution of the CTL hierarchy after the primary response and following successive in vivo restimulations with the same tumor cell line. Longitudinal analyses were performed by analyzing the frequencies of CW3- and PIEm-specific T cells at various time points: peak of the primary response, memory, peak of the secondary response, secondary memory, and third boost (Fig. 5). The evolution of the CTL hierarchy was followed between two successive phases of the immune response in several individual mice. In addition, three mice were analyzed at all time points mentioned (Fig. 5A). As shown in Fig. 5B, the CTL hierarchy evolved similarly in all tested animals. The ratio of anti-CW3 to anti-PIEm T cells among the memory pool was very similar to that observed in the postprimary memory T cells pool. On the average, the ratio of

Table I. Clonal composition of CW3- and PIEm-specific T cell populations

<table>
<thead>
<tr>
<th>CDR3β Sequence (nt)</th>
<th>CDR3β Sequence (aa)</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV10-BJ.12</td>
<td>AGT CTT GCC GAC TAC</td>
<td>S1GSDY</td>
</tr>
<tr>
<td>AGC ACA GCC TCC GAC TAC</td>
<td>STGSDY</td>
<td>2/11</td>
</tr>
<tr>
<td>AGC ACA GCC CTC GAC TAC</td>
<td>STGQDY</td>
<td>1/11</td>
</tr>
<tr>
<td>AGC TCC GCC TCC GAC TAC</td>
<td>SPGSDY</td>
<td>1/11</td>
</tr>
<tr>
<td>AGC CCA GCC GGA AAC TAC</td>
<td>SPQGNY</td>
<td>1/11</td>
</tr>
<tr>
<td>BV1-BJ.2.5</td>
<td>AGC ACA ACA ATT AAC CAA GAC ACC CAG</td>
<td>SQTINQDTQ</td>
</tr>
<tr>
<td>AGC CAG ACA ATT AAC CAA GAC ACC CAG</td>
<td>SQTINQDTQ</td>
<td>3/27</td>
</tr>
<tr>
<td>AGC CAG ACA TTT AAC CAA GAC ACC CAG</td>
<td>SQTINQDTQ</td>
<td>2/27</td>
</tr>
<tr>
<td>AGC CAA ACA GTT AAC CAA GAC ACC CAG</td>
<td>SQTINQDTQ</td>
<td>2/27</td>
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<tr>
<td>AGC CAA ACA TTT AAC CAA GAC ACC CAG</td>
<td>SQTINQDTQ</td>
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<tr>
<td>AGC CAG ACC ATT AAC CAA GAC ACC CAG</td>
<td>SQTINQDTQ</td>
<td>1/27</td>
</tr>
<tr>
<td>AGC CAG ACA GTT AAC CAA GAC ACC CAG</td>
<td>SQTINQDTQ</td>
<td>1/27</td>
</tr>
</tbody>
</table>

DBA/2 mice were immunized i.p. with P815mCW3-B7. At day 12, cDNA were prepared from CD8⁺ splenocytes. BV10-BJ1.2 and BV1-BJ2.5 PCR products were cloned and sequenced. Shown are the sequences obtained from a representative individual mouse.

![Figure 4](http://www.jimmunol.org/)
Selective expansion of CW3-specific T cells during secondary responses. A, Frequencies of CW3- and P1E-specific T cells in the PBLs of individual mice were determined using K^d- peptide tetramers at various time points following successive immunizations with P815 mCW3-B7 (naive, day 0; primary, day 12; memory, day 75; secondary, day 82; secondary memory, day 140; third boost, day 147). Three animals were analyzed at all time points. Results corresponding to a representative mouse are shown. B, Evolution of the CTL hierarchy after the primary response and following successive immunizations with P815 mCW3-B7 in individual animals. The CTL hierarchy (as determined by the ratio of anti-CW3 to anti-P1E T cells) was analyzed between two successive phases of the immune response in several individual mice. Connecting lines indicate values from the same mouse. C, The selective expansion of CW3-specific T cells is independent of the ratio of CW3- to P1E-specific T cells in the memory pool. Naive DBA/2 mice were immunized with the P815 tumor cells transfected with the complete HLA-CW3 molecule. The primary response against this tumor is largely dominated by CW3-specific T cells, and this dominance is also apparent in the pool of memory T cells. Six months after primary immunization, these mice were boosted by i.p injection of P815 mCW3-B7 tumors. CW3- and P1E-specific T cells were quantified 1 day before the boost (postprimary memory), at the peak of the secondary response (day +7), and 2 mo after the secondary response (postsecondary memory).

Anti-CW3 to anti-P1E decreased 4 ± 1.5-fold, strongly suggesting that most expanded CW3 secondary effectors did not enter the secondary memory pool. Finally, CW3-specific T cells were also preferentially expanded following a third tumor injection (Fig. 5B).

To assess whether this selective expansion of CW3-specific T cells during recall responses was dependent on the relative frequency of anti-CW3 memory cells, we used a closely related tumor system to generate very large amounts of CW3-specific memory cells. We took advantage of the pattern of response of DBA/2 mice immunized with a P815 cell line transfected with the complete HLA-CW3 molecule. The primary CTL response against this tumor has been extensively studied and was shown to be mainly focused on the CW3 epitope (23). The reason for CW3 dominance in this response is not clear; one possibility is that the lack of B7 molecule on this tumor cell line diminishes the response against P1E. We immunized DBA/2 mice with this tumor. Six months after tumor rejection, CW3-specific T cells largely dominated the memory T cell pool as shown in Fig. 5C (CW3:P1E ratio, 9.0 ± 4.0). Thus, the contribution of anti-CW3 relative to that of anti-P1E T cells was much stronger in the memory pool induced by P815-HLA-CW3 than in that produced by P815 mCW3-B7 tumors. CW3- and P1E-specific T cells were quantified 1 day before the boost (postprimary memory), at the peak of the secondary response (day +7), and 2 mo after the secondary response (postsecondary memory).

The extent of in vitro expansion of each epitope-specific memory population is modulated by epitope density

To gain insight into the mechanism ruling the selective expansion of CW3-specific T cells during recall responses, we analyzed the ability of memory cells to expand in vitro when stimulated with the tumor. To follow the proliferative history of memory cells, splenocytes from immune mice (4 mo post-P815 mCW3-B7 immunization) were labeled with CFSE and cultured in the presence of P815 mCW3-B7 tumor cells. Using this approach, it was possible to compare the in vitro proliferation of P1E- and CW3-specific T cells in the same culture. At various time points, cell populations were analyzed by flow cytometry using K^d-peptide tetramers. Although memory cells did not expand when cultured alone, both CW3- and P1E-specific T cells divided in the presence of the tumor, as shown by a loss of CFSE content (Fig. 6A). On day 3, all CW3-specific T cells had undergone several cell divisions, whereas a large fraction (generally >50%) of the initial P1E-specific T cell population had not (Fig. 6A). We calculated on the basis of CFSE content that on day 3 anti-CW3 T cells had undergone, on the average, one additional round of cell division compared with anti-P1E T cells (Fig. 6A and data not shown). Moreover, the preferential expansion of CW3-specific T cells was reflected by a progressive contraction of the secondary response (Fig. 5C), suggesting that most anti-CW3 secondary effectors did not contribute to the postsecondary memory.

In summary, whatever their relative contribution to the antitumor memory pool, CW3-specific T cells are selectively expanded in vivo during the secondary response to P815 mCW3-B7.

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In summary, whatever their relative contribution to the antitumor memory pool, CW3-specific T cells are selectively expanded in vivo during the secondary response to P815 mCW3-B7.

The extent of in vitro expansion of each epitope-specific memory population is modulated by epitope density
increase in the CW3:P1E ratio over time, and on day 12, the vast majority of T cells were CW3 specific (Fig. 6A).

To determine how epitope density at the surface of the tumor cells impacts on T cell expansion, we cultured memory cells in the presence of different peptide-pulsed tumors. K\(^d\)-CW3 and K\(^d\)-P1E complexes are both displayed on P815 mCW3-B7 cells. We therefore pulsed this cell line with either the CW3 or the P1E peptide to increase its density at the surface of the tumor cells. As shown in Fig. 6B, increasing one epitope density resulted in a greater expansion of the associated epitope-specific population. Unlike stimulation with unpulsed tumor, P1E-pulsed P815 mCW3-B7 cells lead to recruitment of virtually all P1E-specific memory T cells, leaving none with a high CFSE content (Fig. 6B). Furthermore, these P1E-specific T cells bear, on the average, 4 times less CFSE after expansion (mean CFSE intensities among P1E-specific T cells, 27 vs 110, respectively), indicating that they underwent two more rounds of cell division.

We therefore conclude that, similar to what was observed in vivo, CW3-specific memory T cells are selectively expanded in vitro when stimulated with tumor cells. Moreover, both P1E- and CW3-specific expansions can be individually regulated by the epitope density displayed at the surface of stimulating cells.

**Discussion**

In the present report we have analyzed the relative contributions of two peptide-specific CD8 T cell populations during the successive phases of a T cell immune response. Primary and secondary responses were found to differ in terms of CTL hierarchy. Moreover, our results show that different mechanisms can control the relative size of peptide-specific T cell populations during a primary and a secondary response.

We used the transfected mastocytoma P815 mCW3-B7, which elicited vigorous CD8 T cell responses mainly directed against two K\(^d\)-restricted epitopes; CW3 and P1E. As previously shown in the related P815-HLA-CW3 system, large numbers of Ag-specific T cells are detected not only in the lymphoid tissues, but also in the PBLs (23). It is thus possible to perform longitudinal analyses and to follow the evolution of the CTL hierarchy during the successive phases of an immune response. MHC-peptide tetrameric complexes allow an accurate quantitation of each epitope-specific population without any in vitro restimulation, a step prone to introduce biases (27). In particular, these approaches have allowed us to track memory cells on the basis of their specificity and their ability to persist for a long time (at least several months) after the initial immunization.
Our initial observation was that distinct syngeneic mice mounted different primary CTL responses with regard to the size of each epitope-specific population. In contrast to other systems where the CTL hierarchy is highly reproducible (10, 11), we report that the ratio of CW3- to P1E-specific populations can vary by a factor of 60 from mouse to mouse. These variations could not have been accounted for by a possible individual variability in the course of the antigenic stimulus with regard to overall Ag load, epitope density, and duration of epitope presentation. Indeed, Ly 5.1/Ly 5.2 chimeric mice showed different CTL hierarchies among Ly 5.1 and Ly 5.2 responding T cells (Fig. 2) even though both T cell populations were facing strictly identical antigenic stimuli.

What dictates the size of each epitope-specific population? It has been proposed that the magnitude of responding populations could be directly proportional to the number of CTL precursors present before immunization and would therefore be inherent to the composition of the naive repertoire. The data presented here suggest that the processes involved in determining the CTL hierarchy are more complex and include some stochastic events. Indeed, aliquots of the same naive T cell pool (which should contain the same number of precursors) can respond differently when transferred into distinct recipients (Fig. 3). Collectively, our results support a "first come, first served" rule, in which the specificity of the first T cell clone encountering the Ag could dictate the CTL dominance. Indeed, we have previously demonstrated that within a peptide-specific population, dominant clones were those that had encountered the Ag at an early time point during the immune response (17). Others have also proposed that differences in the timing of recruitment of individual clones could account for the variability in the lytic activity directed against several P815 Ags (26). Importantly, in the present report both CW3 and P1E populations were found to be predominantly comprised of the progeny of one or two clonotypes (Table I), indicating that the number of distinct clones recruited does not account per se for the magnitude of the specific CTL population. Finally, evidence that T cell clones are not primed synchronously upon in vivo immunization has been presented in several reports in which the fate of adoptively transferred TCR Tg T cells was followed using CFSE. In these studies it was found that after 3 days the number of cell divisions undergone by each of these individual monoclonal T cells varied from zero to six (28–31).

Precursor frequencies may, however, impact on the average contribution of each epitope-specific population when large numbers of animals are analyzed. In this respect we observed that the P1E-specific response was dominant in 80% of the mice analyzed. Therefore, under the hypothesis that the primary response follows a "first come, first served" rule, our results indicate that in a naive DBA/2 mouse the probability that an eligible precursor specific for P1E meets and responds to the antigenic stimulus is 4 times higher than the probability for CW3 precursors. These data further suggest that a high precursor frequency in the naive repertoire may in some instances favor CTL dominance by increasing the probability of an early encounter with the Ag.

The most intriguing observation in this study is the difference in the regulation of CTL hierarchy during a primary and a secondary response. As discussed above, while a certain degree of randomness was observed in the primary CTL dominance, expansion of the long-lived memory T cells upon restimulation was selective, with a preferential proliferation of the CW3-specific memory subset. Interestingly, this phenomenon occurs independently of the relative frequency of CW3-specific T cells in the memory pool (before the secondary stimulation), since it has been observed in mice displaying a CW3:P1E ratio among memory T cells ranging from 0.1 to 10 (Fig. 5, B and C). In approximately half the mice analyzed, this selective expansion led to an inverted dominance between the primary and the secondary response. While in other systems the CTL hierarchy observed in the secondary T cell response directly reflected that observed in the primary burst, this was not the case in our system (10, 11). Similar results were also observed in vitro (Fig. 6). It should be noted that reports that have quantified or analyzed memory T cell populations after in vitro restimulations might require a careful reassessment, since our results illustrate that all epitope-specific populations do not always expand equally in culture.

The basis of the epitope-specific expansion of memory cells is not completely clear, and it is important to consider these results in light of the recent reports that have analyzed primary and secondary T cells specific for a single epitope. In two independent systems, a selective expansion of particular specific T cell clones has been observed together with an overall increase in TCR affinity when memory cells expand into secondary effectors (32, 33). One possibility would be that CW3-specific T cells display, on the average, higher TCR affinities compared with anti-P1E CTLs. However, the tetramer staining intensities of memory cells, which were shown to reflect TCR affinity in several instances (34, 35), do not support this hypothesis. If different, the staining intensity of memory P1E-specific T cells is slightly higher than that of anti-CW3 T cells (data not shown). In addition, comparing TCR affinities is not completely relevant in our situation, since we are considering two epitopes possibly presented at different densities. A more relevant parameter should be the overall avidity of the memory populations, which depends on peptide densities. Because the faster kinetics of the secondary response are likely to be reflected by a rapid reduction of the overall Ag load, some epitopes (in our case, the P1E epitope) could be present in limited amounts and during a time period too short to drive the expansion of the whole memory pool. This view is also supported by our observation that delivering high Ag amounts increases both the number of dividing T cells and the average number of cell divisions undergone (Fig. 6B). Therefore, in vitro results support the idea that the relative amount of each epitope dictates the hierarchy of T cell expansions during the secondary response. The fact that this is not the case in the primary T cell response could be due to the overall antigenic load reaching a threshold over which differences in epitope densities no longer impact on the extent of T cell expansion.

We also observed in all analyzed mice that the secondary expanded anti-CW3 T cells are not fully transmitted to the secondary memory pool (Fig. 5, compare secondary and postsecondary). Although the primary burst determines the frequency of memory populations, this is not the case during recall responses. In our experimental system the staining of T cells with MHC-peptide tetramers at the peak of the secondary response does not discriminate between resting memory cells and the progenies of expanding memory cells. Nonetheless, we observed that the preferential expansion of CW3 memory cells upon secondary responses is associated with an inefficient entry in the postsecondary memory compartment. Therefore, progenies of expanding memory cells have a small, if any, contribution to the secondary memory pool.

CTL responses rely upon the expansion of T cell populations that differ in specificity and size. Numerous parameters can impact on the magnitude of the different epitope-specific populations during primary responses (1, 2). The present study shows that extensive differences exist in the regulation of the CTL hierarchy during the successive phases of an immune response. In the light of these results, we propose that quantitative and qualitative changes between naive and memory cells result in differences in Ag clearance. Primary and secondary immunizations would then result in substantially different concentrations of Ag and different durations.
of epitope presentation during the period of T cell expansion. In this respect, some memory cells may face low to limiting amounts of Ag during secondary responses and may be subjected to enhanced competition processes. This could explain why some peptide-specific T cells successfully expand upon primary immunizations but fail to do so during secondary responses.

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References