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# A Class I MHC-Restricted Recall Response to a Viral Peptide Is Highly Polyclonal Despite Stringent CDR3 Selection: Implications for Establishing Memory T Cell Repertoires in “Real-World” Conditions<sup>1</sup>

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In this study, we analyze the recall response to influenza A matrix peptide M1(58-66) restricted by HLA-A2 in one individual and find a strict CDR3 selection as well as a high degree of polyclonality. The TCR  $\beta$ -chain repertoire of memory T cells specific for this Ag system has been shown previously to be constrained by the use of the BV17 family and the <sup>1</sup>/sRS<sup>A</sup>/<sub>S</sub> amino acid motif in the CDR3 region. Our sequence analysis of BV17 TCR from a CTL line showed the repertoire to be highly polyclonal, as 95 distinct CDR3 sequences (clonotypes) were identified expressing this CDR3 motif. The clonotype frequencies showed a power law distribution with an extensive low-frequency tail. The clonotypes present in the high-frequency component of the distribution could be measured directly in the PBMC. This measurement showed that the relative frequencies of these clonotypes before stimulation were similar to their frequencies after culturing. Analysis of short-term cultures showed that the responding clonotypes have a similar ability to proliferate, which is independent of TCR  $\beta$ -chain CDR3 sequence or precursor frequency. These data indicate that the memory T cell repertoire is composed of a surprisingly diverse set of T cell clonotypes with a limited potential for expansion. We propose that the high-frequency component represents T cells that have existed the longest. In keeping with this hypothesis, these clonotypes were measured over a 2-year period, during which their precursor frequency did not change. *The Journal of Immunology*, 1998, 160: 2842–2852.

Since the classic experiments of Zinkernagel and Doherty (1) describing the phenomenon of MHC restriction, the exact nature of the T cell-mediated immune response has been studied from the point of view of the specificity of Ag recognition. A large body of literature has been generated showing examples of how T cells can recognize specific Ag in a MHC-restricted manner. Advances in the understanding of Ag processing (2, 3) together with the solution of MHC (4), TCR (5), and MHC-Ag-TCR structures (6, 7) have further elucidated how the immune system recognizes foreign Ag. However, an immune response is characterized not only by the specific selection of distinct responsive T cells, but also by the generation of a repertoire that can act as effector and as memory for subsequent Ag encounters.

Historically, repertoire analysis has been based on the analysis of T cell clones generated after in vitro stimulation of the memory cells (8–10). This approach is effective in the identification of the TCR  $\alpha$ - and  $\beta$ -chain CDR3 motifs that define the affinity of the MHC-Ag-TCR interactions as well as providing data about the clones' functional activity (lymphokine production, cytotoxicity, etc). However, T cell cloning methods are limited by the number of clones generated and the possibility that those clones may not be representative of the general immune response.

The primary immune response to a particular Ag is mediated by the number of naive T cells with TCRs specific to the Ag-MHC complex and their relative levels of affinity. The extent of proliferation of a given T cell is likely to be a function of its affinity and depends upon help in the form of cell contact or soluble factors. Current investigations focus on the identification of the selective factors that determine which Ag-stimulated T cells become memory T cells, what influences the life-span of those cells, and the complexity of the memory T cell repertoire.

Our understanding of the nature of the memory, or recall, immune responses is less complete. However, progress has been achieved recently in the investigation of the peripheral selection of the Ag-specific T cell repertoires. For instance, it has been shown that MHC class II-restricted memory response is mediated by a limited number of T cell clones. In a study published by McHeyzer-Williams and Davis (11), I-E<sup>k</sup>-restricted recall response to pigeon cytochrome *c* in situ was mediated by a restricted repertoire of T cell clones. Some of those clones increased in frequency up to 1200-fold. A similar result has also been seen in MHC class I-restricted memory response (12).

We are interested in the nature of the memory T cell repertoire, its generation, and its remobilization after restimulation by the

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same or a similar Ag. The main question we address here is: how complex is the memory T cell repertoire in humans? Since the influenza A matrix peptide M1(58-66) is able to generate a finely honed CTL response in the majority of HLA-A2-positive individuals, it was chosen as the Ag system. This response is mediated by CD8<sup>+</sup> T cells expressing the TCR  $\beta$ -chain of BV17 family and utilizes an I-R-S amino acid motif in the CDR3 region (13, 14). In one study, 35 CTL clones were generated with unique CDR3 bearing this motif (14). Three of four CTL from one individual had identical CDR3 encoded in different ways (14). Because T cell cloning may be incomplete or biased, T cell lines were analyzed using molecular genetic techniques. In this study, the term "clonotype" refers to a "clone," which is still a part of a more complex responding repertoire and which is identified by its unique TCR BV CDR3 nucleotide acid sequence. The breadth of the response in CTL lines was analyzed using CDR3 length analysis, which showed that by the time the CTL response was well established, CD8<sup>+</sup>, BV17-expressing T cells utilizing two CDR3 lengths predominated in the culture. The BV17 response was further examined by sequence analysis of the CDR3 region. A large number of clonotypes was identified. This complex repertoire is described in terms of clonotype frequencies, CDR3 sequence motifs, and proliferative capacity. The results are discussed in terms of how complex repertoires may be generated.

## Materials and Methods

### *Influenza virus M1(58-66)-specific, HLA-A2.1-restricted CTL*

PBMC were collected from buffy coat cells from an HLA-A2.1 blood donor using Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ). Three blood samples were used. The triplicate CTL lines used for sequence analysis were generated from PBMC collected in 1994. PBMC for the 7-day culture and clonotype precursor frequency analysis were obtained 11 and 23 mo after those used in the first series of CTL.

Triplicate CTL lines, referred to as CTL-1, CTL-2, and CTL-3, were established as previously described (15). PBMC were cultured at a concentration of  $2 \times 10^6$  cells/2 ml in the presence of 0.5 mg/ml of influenza A matrix peptide M1(58-66) in 24-well plates for 7 days. Recombinant IL-2 (10 U/ml) was added on day 4. The primed cells were restimulated weekly in the presence of rIL-2 (10 U/ml) and an equal number of irradiated autologous PBMC (3000 R) or HLA-A2.1-matched B cell lines (B-LCL) (10,000 R) that had been precoated with M1(58-66) at 0.5 mg/ml for 1 h. Recombinant IL-2 was added on day 4 of each additional week culture.

Cytotoxic activity was measured starting with week 3 of culture using a standard <sup>51</sup>Cr-release assay as previously described (15). The target cells included the B-LCL JY (HLA-A2, -B7), and Hmy2.C1R individually transfected with the genes encoding HLA-A2, HLA-B7, or HLA-B27.

Short-term lines were established as described above for generating lines, except that culture was for only 7 days. Samples were collected at days 2, 4, 6, and 7.

### *Immunomagnetic cell separation*

Immunomagnetic cell separation was used to isolate a pure CD8<sup>+</sup> T cell subpopulation from the three CTL lines after 5 wk of M1(58-66) stimulation. mAb OKT8 (Becton Dickinson, San Jose, CA) and Dynabeads M-450 coated with goat anti-mouse IgG (DynaL A.S, Oslo, Norway) were used according to manufacturer recommendations. Before application, mAb was titrated to saturate the CD8<sup>+</sup> T cells.

### *CDR3 spectratyping*

Total RNA was prepared by homogenizing the cells in guanidinium isothiocyanate and pelleting through a CsCl cushion. cDNA were generated by conversion of 2  $\mu$ g of the total RNA into cDNA using oligo(dT) as a primer for reverse transcription in a 100- $\mu$ l cDNA synthesis reaction.

BV genes were amplified from cDNA using 24 BV family-specific primers either in pairs or singly. The sequence and use of <sup>32</sup>P- or fluorochrome-labeled TCR BC region-specific primers have been previously described (16). The use of TCRBJ region-specific primers and their sequences have also been described (17). Thirty rounds of amplification were routinely performed.

Ten microliters of the final PCR volume were subjected to electrophoresis on a 5% polyacrylamide sequencing gel and the ensuing bands visual-

ized by autoradiography or fluorescence detection (FluorImager 575; Molecular Dynamics, Sunnyvale, CA). Band number is a measure of repertoire complexity. Band intensity can reflect both complexity (many different T cells with TCR of the same CDR3 length) and TCR quantity (expansion of one or more T cells with TCR of the same CDR3 length). A detailed description of the protocols can be found in Reference 18.

### *PCR product cloning, sequencing, and colony counting*

cDNA synthesized from the CTL RNA samples was PCR amplified with a BV17 family-specific primer containing a *Pst*I restriction site (5'-GCCCTGCAGATAGTAAATACTTTTCAG-3') and a C $\beta$ -specific primer containing a *Bam*HI restriction site (5'-GAGCCATCAGAAGCAGAGATCTGATCCCCCA-3'). After digestion, amplification products were cloned into the corresponding sites of the plasmid vector pGEM-3Zf(+) (Promega, Madison, WI). Before the cDNA cloning, precautions were taken to avoid saturation of the PCR product by amplifying serially diluted cDNA in a pilot experiment. cDNA diluted 32-fold, which was still in the linear amplification range, was used for TCR- $\beta$  plasmid cloning. White colonies containing cDNA inserts (176 colonies from CTL-1 and 118 clones from CTL-2 cDNA minilibraries) were picked and grown on nitrocellulose membranes. All colonies tested positive with a BV17 family sequence-specific oligonucleotide probe (BV17-SSOP).<sup>5</sup>

The bacterial colonies, plated on nylon membranes, were screened using an iterative process that consisted of repetitive sequencing and hybridization. Initially, nine colonies were randomly chosen for sequencing using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (ABI, Foster City, CA). Clonotype-specific oligonucleotide probes (cl-SSOPs) (14-mers) were generated from those sequences by choosing the nucleotides corresponding to the CDR3 region and including a few bp from the 3'-end of the BV17 region.

Membranes were hybridized overnight with <sup>32</sup>P-labeled cl-SSOPs at a temperature ranging from 37° to 40°C (depending on the cl-SSOP sequences), washed twice in the first washing solution (3 $\times$  SSC, 10 $\times$  Denhardt solution, 5% SDS, 70 mM phosphate buffer, pH 7.0) and twice in the second washing solution (1 $\times$  SSC, 1% SDS). After overnight autoradiography, probe-specific colonies were identified and counted. After each hybridization cycle, the cl-SSOPs were stripped from the nitrocellulose membranes by boiling in 1% SDS.

Controls on the filters included the original colony from which the sequence was derived and colonies with similar but not identical sequences (positive and negative controls, respectively). Hybridization and wash conditions were optimized for each probe. In case a hybridization result was inconclusive (low signal to noise), the colony was sequenced.

Colonies that did not hybridize with the cl-SSOPs were chosen for the next round of sequencing. After approximately half the colonies had been identified by this procedure, the remaining colonies were simply sequenced. All 294 TCR BV17-positive colonies (176 from CTL-1 and 118 from CTL-2 minilibraries) were analyzed by the procedure.

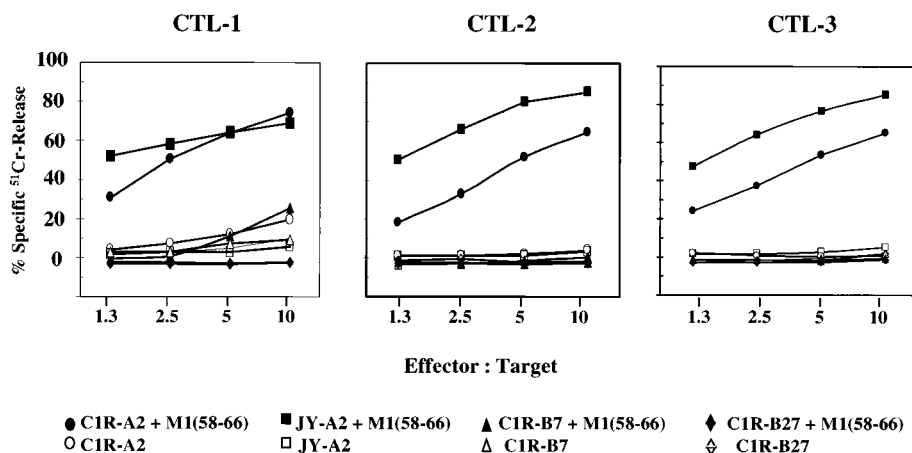
### *Hybridization of the amplified cDNA (clonotyping)*

cDNA were PCR amplified using BV17 family-specific- and BJ2.7-specific primers as described above. To avoid saturated PCR reactions that may change the proportion between T cell clonotypes sharing a CDR3 length, serially diluted cDNAs were PCR amplified. A cDNA dilution within the linear range of increase in PCR band intensity was used for PCR amplification. For the PCR, 1  $\mu$ l of the cDNA synthesis reaction mixture was used undiluted for 0 day analysis, and diluted 8-fold and occasionally 16-fold for the short-term time course analysis. Ten microliters of PCR-amplified CTL cDNA and serially diluted plasmid DNA PCR product were loaded on a 5% denatured sequencing gel, run for 1 h, electroblotted from the gel onto a nitrocellulose membrane (Micron Separation Inc., Westboro, MA), and immobilized.

Membrane-bound DNA was hybridized with <sup>32</sup>P-labeled cl-SSOP overnight, and washed twice in first washing and twice in second washing solutions as described above. Only cl-SSOPs that provided good resolution between specific DNA and nonspecific DNA hybridization were used. A Storage Phosphor Screen (Molecular Dynamics) was exposed to the hybridized membrane overnight and screened on a PhosphorImager SP 400 (Molecular Dynamics). ImageQuant software was used to visualize the CDR3 bands and to evaluate their radioactivity. After stripping, the membranes were rehybridized overnight with BV17-SSOP at 50°C and washed at 50°C as described above. Clonotype-specific CDR3 band intensity was

<sup>5</sup> Abbreviations used in this paper: BV17-SSOP, TCR BV17 family-specific oligonucleotide probe; cl-SSOP, clonotype-specific oligonucleotide probe; RT, reverse transcription; RF, relative frequency.

**FIGURE 1.** Cytotoxicity analysis of three independent long-term CTL lines derived from the HLA-A2.1<sup>+</sup> individual. Each panel shows the results for one cell line. Target cells were JY (HLA-A2, -B7, ■ and □) or C1R cells individually transfected with the genes for HLA-A2 (● and ○), HLA-B7 (▲ and △), or HLA-B27 (◆ and ◇). <sup>51</sup>Cr-labeled target cells were either prepulsed (filled symbols) with M1(58-66) peptide (0.5 mg/ml, 1 h at 37°C) or not treated with peptide (empty symbols). Effector and target cells were incubated together at the indicated E:T ratio for 4 h at 37°C, and % <sup>51</sup>Cr-release calculated.



evaluated on a PhosphorImager SP 400 after hybridization with cl-SSOP and BV17-SSOP as described above. The intensity patterns of the fluorescent spectratypes generated before electroblotting looked identical to the hybridization intensity with BV17 SSOP. This serves as a control for the efficiency of electroblotting.

The relative frequency (RF) of an individual T cell clonotype was evaluated as:

$$RF = (\text{cl-SSOP cDNA cpm} \div \text{VB-17-SSOP cDNA cpm}) \times K,$$

where  $K$  is the probe correction factor, defined as:

$$K = \text{VB-17-SSOP plasmid cpm} \div \text{cl-SSOP plasmid cpm}.$$

## Results

### *Cytotoxic T cell repertoires responding to influenza A matrix peptide 58-66 are composed of BV17<sup>+</sup>CD8<sup>+</sup> T cells*

Since the main goal of the present study is to evaluate the complexity of the memory T cell repertoire, our experimental model should satisfy several criteria. First, T cell clonotypes whose existence and frequencies we would like to identify should be Ag specific. Second, the experimental method should exclude procedures that would skew or incompletely sample the repertoire. Third, the experimental model should be sensitive enough to detect low-frequency clonotypes.

To this end, we utilized the well-studied HLA-A2-restricted response to influenza A matrix peptide M1(58-66) and studied the response in short-term cultures using molecular genetic methods for repertoire analysis. Previous work has shown that the predominant M1(58-66)/HLA-A2.1-specific cytotoxicity is mediated by CD8<sup>+</sup> effector cells expressing the TCR  $\beta$ -chain of the BV17 family, utilizing an I-R-S amino acid motif in the CDR3 region (13, 14). This motif was found in a number of individuals and, in some cases, T cell clones from the same individual were obtained with identical CDR3 amino acid sequences encoded for in a number of ways. Thus, this combination of Ag and MHC promised to be a good candidate for analysis.

Three independent CTL lines (CTL-1, -2, and -3) were established from the PBMC of an HLA-A2<sup>+</sup> donor and were cultured for 5 wk to eliminate NK-mediated activity. After 5 wk in culture, the lines were shown to be Ag and MHC specific as measured by <sup>51</sup>Cr-release assays (15). The CTL only recognized target cells that expressed HLA-A2.1 and had been pulsed with M1(58-66) or HLA-A2.1 transfectants pulsed with the same Ag (Fig. 1).

The T cell repertoire present in the CTL lines was studied by CDR3 length analysis. This is based on resolving of the TCR  $\beta$ -chain mRNA reverse transcription (RT)-PCR amplification products on acrylamide gels. Band intensity can reflect both com-

plexity (many T cells with different TCR but with the same CDR3 length) and quantity (expansion of one or more T cell clonotypes with the same TCR) (18).

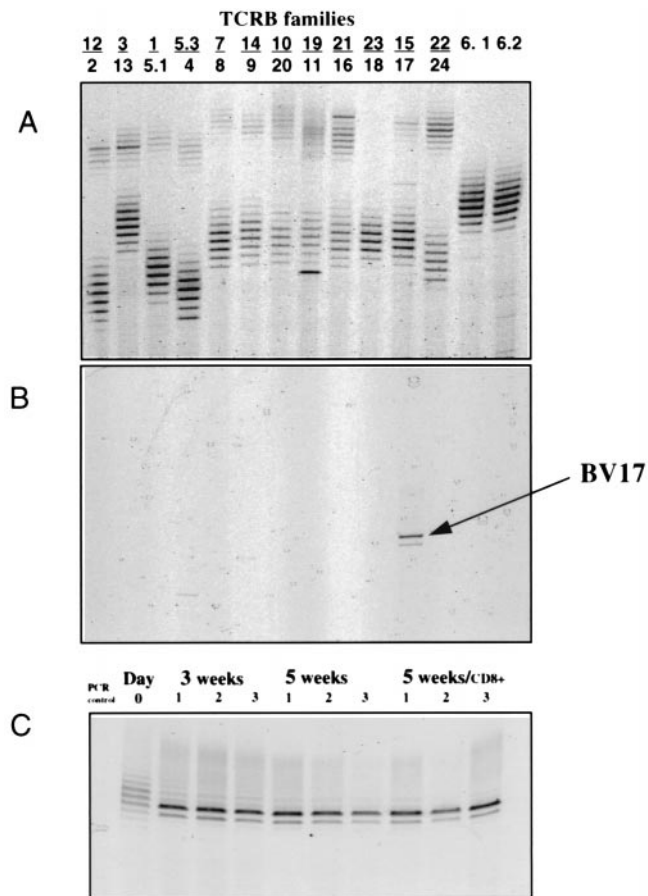
The TCR  $\beta$ -chain repertoires of the PBMC (day 0) and CTL (5 wk with M1(58-66) stimulation) are shown in Figure 2, *A* and *B*. From the complex pattern seen at day 0, the repertoire narrows to BV17<sup>+</sup> T cells with two CDR3 sizes. Indeed, when the cDNA was diluted up to 256-fold, the two CDR3 bands within BV17 were still easily visible, whereas the CDR3 from other BV families were almost nonexistent (Fig. 2*B*). Moreover, the expansion of the M1(58-66)-restricted BV17<sup>+</sup>CD8<sup>+</sup> T cells was observed in all three independent CTL lines and in the CD8 subset of these lines (Fig. 2*C*). Occasional bands in other families (see BV4 lane, Fig. 2*B*) were not observed reproducibly. These results are in keeping with T cell cloning data that indicated the importance of the BV17 family utilizing two CDR3 lengths in these responses (14). Thus, using CTL lines, we were able to expand M1(58-66)-specific CD8<sup>+</sup> T cells and focus our study on only the BV17 family.

### *Repertoires responding to the influenza A matrix peptide are complex*

To evaluate the TCR repertoire complexity of the responding memory cells, mRNA from CD8<sup>+</sup> T cells from line CTL-1 and CTL-2 at week 5 (Fig. 2*C*) were used for cDNA cloning. Generation of cDNA clones from two independent CTL lines was used to minimize effects from the culturing procedure itself. The cDNA was diluted 32-fold before amplification to insure that the PCR was not saturated and that clone frequency would represent cDNA frequency. At the dilution used, the resolution of the CDR3 region PCR products for BV17 consisted of only two bands and looked identical to the 256-fold dilution shown in Figure 2*C* (data not shown). The 294 bacterial colonies (176 from CTL-1 and 118 from CTL-2) that gave positive hybridization signals with a <sup>32</sup>P-labeled BV17 family-specific oligonucleotide probe were analyzed. The first stages of the analysis utilized a recursive hybridization protocol to identify already-sequenced colonies, as described in *Materials and Methods*. The final 50% of the colonies were sequenced directly. In this way, the CDR3 of all 294 colonies were determined and 95 unique sequences, representing 95 clonotypes, were identified (Table I).

### *The characteristics of the TCR of the responding repertoire*

Analysis of the nucleotide sequences for the 95 clonotypes showed that 65% (62 of 95) of the clonotypes used JB2.7 (Table 1). Among these, five predominant CDR3 amino acid motifs were



**FIGURE 2.** T cell repertoire analysis based on CDR3 length heterogeneity. *A*, TCR BV repertoire generated from PBMC (day 0 of culture) using a labeled C region primer and two BV family-specific primers per PCR. The pair of co-amplified families analyzed is identified above each lane with the upper number referring to the family migrating higher in the lane. cDNA was not diluted before PCR amplification. *B*, TCR- $\beta$  repertoire of the CTL stimulated for 5 wk with the M1(58-66) peptide as described in *Materials and Methods*. The lane descriptions for *A* extend to this gel. cDNA was diluted 256-fold before PCR amplification. The arrow points to the BV17 portion of the 15/17 lane. *C*, Analysis of TCR BV17 repertoire of the triplicate CTL lines after different times of culture. Lanes with PCR of CTL-1, CTL-2, and CTL-3 are labeled as 1, 2, and 3, respectively. The analysis of PBMC is also shown as day 0. Week 5 cultures were also fractionated into CD8<sup>+</sup> cells (5 wk/CD8<sup>+</sup>). Amplification was with a BV17 primer alone. Before PCR amplification: cDNA from week 3 cultures were diluted 32-fold. cDNA from 5 wk total and CD8<sup>+</sup> cultures were diluted 256-fold. cDNA from day 0 were undiluted.

observed. The sequence I-R-S-S was encoded in various ways 26 times; V-R-S-S encoded 7 times; and I-R-S-A, S-R-S-S, and T-R-S-S encoded 6, 4, and 4 times, respectively (Table I). Three of these motifs have been shown to be used by M1(58-86)-specific T cell clones, although some of these came from different individuals (14). Eleven other CDR3 sequences were identified in the BJ2.7 group, some of them being encoded for in two or three different ways (Table I).

The second most common BJ region utilized was BJ1.2, which was associated with the CDR3 amino acid motif I-G-S-Y. We observed this motif encoded five different ways as well as five other I-G-S-Y-related sequences. TCRs expressing this BJ combination had CDR3 that was shorter by one amino acid. These T cells were responsible for the 3-bp smaller band observed in the spectratype analysis (Fig. 2, *B* and *C*). This same combination has also been observed in CTL clones (14).

In addition to the sequenced BJ2.7<sup>+</sup> and BJ1.2<sup>+</sup> clonotypes, we identified 23 other non-BJ2.7<sup>+</sup> clonotypes encoding  $\beta$ -chains with I-R-S-like CDR3 amino acid motifs. As observed for the BJ2.7 clonotypes, these sequences show homology with, or are identical to, ones previously described in clones generated from a number of individuals (14).

#### *Clonotype distribution in the responding repertoire*

The clonotype frequency distribution provides more information as to the nature of the repertoire. The frequency was measured by counting the number of bacterial colonies with the same sequence. This procedure assumes that the bacterial cloning steps do not greatly skew the distribution. The results are shown in Table I (*last column*) and in graphic form in Figure 3. In Figure 3, the clonotypes are plotted in decreasing contribution to the repertoire, and each clonotype is identified on the x-axis. The distribution can be best approximated by a power law. A characteristic of this kind of distribution is the extensive low-frequency tail. This can be easily seen in Figure 3.

Indeed, there is a higher-frequency component in the distribution that may represent clonotypes that expanded as the result of selection. Because the selection could be a function of the J region or the CDR3 sequence, it was of interest to correlate frequency with these two parameters. If certain BJ or CDR3 sequences have been selected for, they should be clustered or over represented in the high-frequency group.

There is a slightly increased proportion of BJ2.7<sup>+</sup> clonotypes in the high-frequency group. If this group is defined as the top 10 clonotypes (Fig. 3), BJ2.7 is present at 80% compared with 65% overall. Similarly, it is slightly under-represented in the low-frequency clonotypes, being present at 53%. This could be indicative of some proliferative advantage of T cells expressing this J region.

On the basis of the above data, we restricted our analysis of the correlation of CDR3 motifs with clonotype frequency to the BJ2.7-bearing clonotypes (filled bars in Fig. 3). While the two highest-frequency clonotypes, 10 and 12, utilize the I-R-S-A motif (star in Fig. 3), other IRSA clonotypes are also found in the medium or low-frequency groups. The same observation can be made for clonotypes with other motifs (IRSS, VRSS, SRSS) or BJ1.2 (IGSY), which are also distributed throughout the different frequency groups. Taken together, our results indicate that there is no specific selection on a subset of the I-R-S CDR3 amino acid motif or on J segment use that could explain the clonotype frequency distribution.

#### *Measuring precursor frequency of select BV17-J2.7<sup>+</sup> clonotypes*

The clonotype diversity and frequency described in the previous section were derived from CTL lines after 5 wk of peptide stimulation. Since culturing could change or skew the distribution, we compared the distribution of the clonotypes in the culture with that in the starting PBMC population. The precursor frequencies of selected clonotypes were determined in the PBMC used for generating the CTL lines by application of a molecular hybridization technique. The clonotypes analyzed represented the high- and mid-frequency components of the repertoire of responding memory T cells, as these gave more reliable hybridization signals.

This was accomplished using cDNA prepared from unstimulated PBMC amplified with BV17- and BJ2.7-specific primers, upon electrophoresis, electroblotting from the gel onto nylon membranes, and hybridization with cl-SSOPs. Subsequently, the membranes were stripped and rehybridized with BV17-SSOP. Three different cDNA concentrations were used to show that the cl-SSOP hybridization signal obtained was proportional to the amount of cDNA analyzed. An example of such an analysis for clonotype 10

Table I. Polyclonality of the responding BV17<sup>+</sup> T-cells specific to M1 (58-66): TCRβ CDR3 nucleotide and amino acid sequences

Clonotype	BV17 <sup>+</sup> Gene 3'-End	N/D/N	BJ Gene 5'-End	CDR3 Amino Acid Motif	No. of Colonies <sup>a</sup>
J2.7-family					
1	GCCAGTAGTAT	TAGGAG	<b>CTCCTACGAGCAGTACTTC</b> <sup>b</sup>	IRSS <sup>c</sup>	14
11	GCCAGTAGTATA	CGGAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	10
94	GCCAGTAGTAT	CAGGAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	10
78	GCCAGTAGTAT	TAGAAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	8
71	GCCAGTAGTATA	AGGAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	3
144	GCCAGTAGTATA	CGAAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	3
32	GCCAGTAG	CATTAGGAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	2
19	GCCAGTAG	CATACGGAGT	<b>TCCTACGAGCAGTACTTC</b>	IRSS	2
22	GCCAGTAG	CATTCCGAGT	<b>TCCTACGAGCAGTACTTC</b>	IRSS	2
90	GCCAGTAGTAT	CAGGTC	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	2
137	GCCAGTAGTAT	CCGGAGT	<b>TCCTACGAGCAGTACTTC</b>	IRSS	2
145	GCCAGTCGTATA	AGGAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	2
49	GCCAGTAGTATA	CGTTCT	<b>TCCTACGAGCAGTACTTC</b>	IRSS	1
50	GCCAGTAGTAT	TAGGAGT	<b>TCCTACGAGCAGTACTTC</b>	IRSS	1
132	GCCAGTAGTAT	TCGGAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	1
141	GCCAGTAGTAT	CCGCTCTAGTG	ACGAGCAGTACTTC	IRSS	1
110	GCCAGTAGTATA	AGAAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	1
140	GCCAGTAGTATA	AGGAGT	<b>TCGGACGAGCAGTACTTC</b>	IRSS	1
148	GCCAGTAGTATA	AGGTCACGCGTG	GAGCAGTACTTC	IRSS	1
147	GCCAGTAGTATA	CGTAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	1
150	GCCAGTAGTAT	TAGAAGT	<b>TCCTACGAGCAGTACTTC</b>	IRSS	1
149	GCCAGTAGTAT	CCGTTC	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	1
151	GCCAGTAGTAT	CCGTAG	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	1
165	GCCAGTAGTAT	TAGGTC	<b>CTCCTACGAGCAGTACTTC</b>	IRSS	1
164	GCCAGTAGTATA	CGAAGT	<b>TCCTACGAGCAGTACTTC</b>	IRSS	1
180	GCCAGTAGTATA	AGGAGT	<b>TCCTACGAGCAGTACTTC</b>	IRSS	1
142	GCCAGTAG	CGTCAGGAG	<b>CTCCTACGAGCAGTACTTC</b>	VRSS	5
25	GCCAGTAGT	GTGCGGAG	<b>CTCCTACGAGCAGTACTTC</b>	VRSS	2
42	GCCAGTAGT	GTCAGGAG	<b>CTCCTACGAGCAGTACTTC</b>	VRSS	1
81	GCCAGTAG	CGTTAGGAG	<b>CTCCTACGAGCAGTACTTC</b>	VRSS	1
57	GCCAGTAGT	GTCCGATC	<b>CTCCTACGAGCAGTACTTC</b>	VRSS	1
156	GCCAGTAGT	GTCCGGTC	<b>CTCCTACGAGCAGTACTTC</b>	VRSS	1
157	GCCAGTAGT	GTCAGGAG	<b>CTCCTACGAGCAGTACTTC</b>	VRSS	1
10	GCCAGTAGTATA	AGGTCAG	<b>CCTACGAGCAGTACTTC</b>	IRSA <sup>c</sup>	47
12	GCCAGTAGTAT	TCGTTCCG	<b>CCTACGAGCAGTACTTC</b>	IRSA	28
130	GCCAGTAGTAT	CCGGAGTG	<b>CCTACGAGCAGTACTTC</b>	IRSA	2
152	GCCAGTAGTATA	CGGTCAG	<b>CCTACGAGCAGTACTTC</b>	IRSA	1
153	GCCAGTAGTAT	TAGATCGGCT	TACGAGCAGTACTTC	IRSA	1
154	GCCAGTAGTAT	TAGATCGCGT	TACGAGCAGTACTTC	IRSA	1
88	GCCAGTAGTA	CTCGGAG	<b>CTCCTACGAGCAGTACTTC</b>	TRSS	1
99	GCCAGTAGTA	CACGATC	<b>CTCCTACGAGCAGTACTTC</b>	TRSS	1
166	GCCAGTAGTA	CCCAGTC	<b>CTCCTACGAGCAGTACTTC</b>	TRSS	1
155	GCCAGTAGTA	CACGCAG	<b>CTCCTACGAGCAGTACTTC</b>	TRSS	1
62	GCCAGTAGT	TCGCGAAG	<b>CTCCTACGAGCAGTACTTC</b>	SRSS <sup>c</sup>	15
27	GCCAGTAGT	TCCCCTTCA	<b>TCCTACGAGCAGTACTTC</b>	SRSS	9
61	GCCAGTAGTA	GCAGGAG	<b>CTCCTACGAGCAGTACTTC</b>	SRSS	5
18	GCCAGTAGT	TCGCGGAG	<b>CTCCTACGAGCAGTACTTC</b>	SRSS	1
35	GCCAGTAGTATA	AGGTCGGGGAC	CGAGCAGTACTTC	IRSG	2
159	GCCAGTAGTAT	CCGATCGGGATT	CGAGCAGTACTTC	IRSG	1
160	GCCAGTAGTAT	TAGGAGCGG	<b>CTACGAGCAGTACTTC</b>	IRSG	1
8	GCCAGTAGT	TCAAGGGC	<b>CTCCTACGAGCAGTACTTC</b>	SRAS	6
56	GCCAGTAGT	TCGCGGAG	<b>CTCCTACGAGCAGTACTTC</b>	SRAS	1
84	GCCAGTAGTAT	GCGTAGCAG	<b>CTACGAGCAGTACTTC</b>	MRSS <sup>f</sup>	1
161	GCCAGTAGTAT	GCGGAG	<b>CTCCTACGAGCAGTACTTC</b>	MRSS	1
102	GCCAGTAGTA	CCAGGGC	<b>CTCCTACGAGCAGTACTTC</b>	TRAS	1
143	GCCAGT	TCTACCAGGTCG	<b>CCTACGAGCAGTACTTC</b>	TRSA	4
5	GCCAGTAGTA	GCCGGTCTG	<b>CCTACGAGCAGTACTTC</b>	SRSA <sup>c</sup>	1
74	GCCAGTAGT	CCCAGAAGCG	<b>CCTACGAGCAGTACTTC</b>	PRSS	1
125	GCCAGTAGT	GGGCGGTC	<b>CTCCTACGAGCAGTACTTC</b>	GRSS	1
146	GCCAGTAGTATA	AGGAGTAC	<b>CTACGAGCAGTACTTC</b>	IRST	2
158	GCCAGTAGTAT	TCGATCGCAAGAG	GAGCAGTACTTC	IRSQ	1
162	GCCAGTAGT	TCCCCTACA	<b>TCCTACGAGCAGTACTTC</b>	SRTS	1

Table I. Continued

Clonotype	BV17 <sup>+</sup> Gene 3'-End	N/D/N	BJ Gene 5'-End	CDR3 Amino Acid Motif	No. of Colonies <sup>a</sup>
<b>J1.2-family</b>					
40	GCCAGTAGT <b>TAT</b>	CGGGAG	<b>CTATGGCTACACCTTC</b>	IGSY	8
167	GCCAGTAGT <b>TAT</b>	CGGCAG	<b>CTATGGCTACACCTTC</b>	IGSY	5
14	GCCAGTAGT <b>TAT</b>	TGGGAG	<b>CTATGGCTACACCTTC</b>	IGSY	2
168	GCCAGTAGT <b>TAT</b>	CGGTTC	<b>CTATGGCTACACCTTC</b>	IGSY	2
169	GCCAGTAAT <b>TAT</b>	CGGCAG	<b>CTATGGCTACACCTTC</b>	IGSY	1
54	GCCAGTAGT <b>A</b>	ACGGGGC	<b>CTATGGCTACACCTTC</b>	NGAY	9
38	GCCAGTAGT <b>TAT</b>	CGGGGTT	<b>TATGGCTACACCTTC</b>	IGVY	2
48	GCCAGCT <b>CGA</b>	CGGGGCT	<b>CTATGGCTACACCTTC</b>	TGLY	1
170	GCCAGTAGT <b>TAT</b>	CGGGGC	<b>CTATGGCTACACCTTC</b>	IGAY	1
171	GCCAGT <b>GGTA</b>	CAGGGAG	<b>CTATGGCTACACCTTC</b>	TGSY <sup>c</sup>	1
<b>J2.3-family</b>					
4	GCCAGTAGT <b>TAT</b>	TCGATCG	<b>GCAGATACGCAGTATTTT</b>	IRSA	1
118	GCCAGTAG	CTCTCGGAGTTC	GATACGCAGTATTTT	SRSS	1
20	GCCAGTAG	CTTCCGATCCA	CAGATACGCAGTATTTT	FRST	1
29	GTCAGTAGT	TCGAGGAGCA	<b>CAGATACGCAGTATTTT</b>	SRST <sup>c</sup>	1
126	GCCAGTAG	<b>CCACAGAAGCA</b>	<b>CAGATACGCAGTATTTT</b>	HRST	1
172	GCCAGTAGT	GGACGGAGCA	<b>CAGATACGCAGTATTTT</b>	GRST <sup>c</sup>	1
173	GCCAGTAGT	CCCAGGAGCA	<b>CAGATACGCAGTATTTT</b>	PRST <sup>c</sup>	1
<b>J2.1-family</b>					
82	GCCAGTAGT <b>TAT</b>	CAGAGCGGGGGCCT	GCAGTTCCTTC	IRAG	2
93	GCCAGTAGT <b>A</b>	CCAGATCAGGAGTCTC	GCAGTTCCTTC	IRAS	1
176	GCCAGTAGT <b>A</b>	CCAGATCAGGAGTCTC	GCAGTTCCTTC	TRSG	1
177	GCCAGTAGT <b>TAT</b>	TCTCTCAGGCGGGCT	GCAGTTCCTTC	ILSG	1
<b>J2.5-family</b>					
7	GCCAGTAGT <b>TATA</b>	AGGAGCGG	AGAGACCCAGTACTTC	IRSR	3
31	GCCAGTAGT	TCCCCTCCCTCC	GAGACCCAGTACTTC	SRSS	1
92	GCCAGTAGT	TCACGTTCCGG	AGAGACCCAGTACTTC	SRSQ	1
<b>J1.5-family</b>					
100	GCCAGTAGT <b>TATA</b>	CGGTCCCAGGAGA	CCCAGCATTTT	IRSQ	4
129	GCCAGTAGT <b>TAT</b>	TCAC	<b>AGCAATCAGCCCCAGCATTTT</b>	IHSN	1
175	GCCAGTAGT	GACCGG	<b>AGCAATCAGCCCCAGCATTTT</b>	QRSN	1
<b>J2.4-family</b>					
77	GCCAGTAGT <b>A</b>	CCCCTCGGGAC	ACATTCAGTACTTC	TRSG	1
178	GCCAGTAGT <b>TAT</b>	CCGTACGGGGG	ACATTCAGTACTTC	IRTG	1
<b>J1.1-family</b>					
76	GCCAGTAGT	CCCCGCTC	<b>GAACACTGAAGCTTTCTTT</b>	PRSN	1
174	GCCAGTAGT <b>TAT</b>	GAGGAGTGGG	ACTGAAGCTTTCTTT	MRSQ	1
<b>J1.4-family</b>					
85	GCCAGTAGT	TGGAGTTCGGTCGAGG	AACTGTTTTTTT	WRSV	1
<b>J2.2-family</b>					
136	GCCAGTAGT <b>TATA</b>	CGTTCGG	<b>ACGGGGAGCTGTTTTTTT</b>	IRSD <sup>c</sup>	2

<sup>a</sup> Number of colonies (294 total) which had the same nucleotide sequence.

<sup>b</sup> The CDR3 length is 9 amino acids as defined by Chothia et al. (23) and encoded as S-<sup>1</sup>/<sub>5</sub>-R-S-<sup>2</sup>/<sub>5</sub>-X-X-X-X except for the BJ1.2 containing CDR3 which are 8 residues. Bold nucleotide sequences were translated into deduced amino acid sequences in CDR3 region as shown on the right side.

<sup>c</sup> TCR $\beta$  CDR3 amino acids used by M1 (58-66) specific T-cell clones, from Lehner et al. (14).

is shown in Figure 4. The hybridization of the electroblotted spectratype is shown in Figure 4A. Figure 4B shows rehybridization with a BV17-SSOP after the clonotype 10 probe was stripped from the membrane.

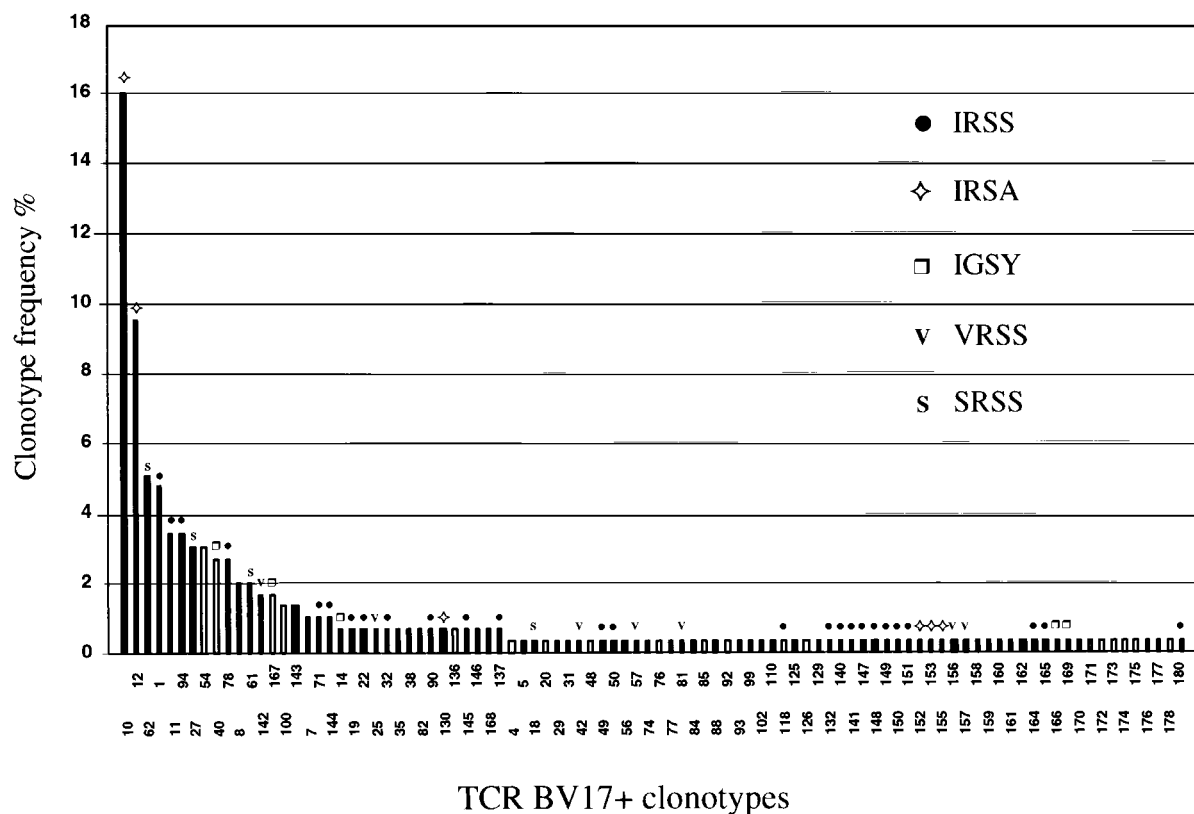
The signal intensity of the hybridization was measured for both probes by phosphorimaging. For the BV17 probe, only the signal corresponding to the correct CDR3 length was measured. The hybridization data included a control composed of the PCR product from the plasmid clone corresponding to the probe used. This could be used to derive a probe correction factor for the two probes used. The probe targets are present in equimolar concentration on the amplified plasmid; thus, any deviations from equivalent hybridization signals represent differential probe efficiencies that must be corrected for. All clonotype frequency data are expressed as the clonotype to BV17 ratio, corrected for probe efficiency (Fig. 4C). This normalization permits comparison of data derived in different experiments with the different probes.

The data for seven clonotypes are shown in Figure 5 (filled bars). The relative precursor frequencies were compared with the

frequencies after 5 wk of stimulation (Fig. 5, empty bars). A few of the clonotypes changed relative position after culture, but they remained close to their original frequency. This indicates that the culturing procedures did not extensively disturb the distribution of the T cell clones with high-precursor frequencies. The precursor frequency of two low-frequency clonotypes (90 and 133) was analyzed but did not give a measurable signal over background (data not shown). It is interesting to note that although one could expect a large deviation between initial frequencies and the frequencies after 5 wk in culture, this was not the case.

#### Measuring *in vitro* kinetics of select BV17-J2.7 clonotypes

The similarity between clonotype precursor frequency distributions and the distribution derived from the 5-wk cultures indicates little difference between proliferation of different clonotypes. *In vitro* proliferation can be viewed as an approximation of *in vivo* activity; therefore, it would be important to test whether the proliferative capacity of most clonotypes is similar. Because long-term cultures can be influenced by multiple variables, we restricted



**FIGURE 3.** Colony frequencies of the T cell clonotypes present in cultures responding to HLA-A2/M1(58-66) peptide stimulation. The TCR BV17<sup>+</sup> cDNA clones are identified on the x-axis. Nucleotide and amino acid sequences derived from the bacterial clones are listed in Table I. The y-axis indicates the relative frequencies as percent of 294 total colonies. Filled bars indicate clonotypes utilizing BJ2.7. White bars represent non-BJ2.7 clonotypes. CDR3 motif utilization for IRSS (●), IRSA (◇), IGSY (□), VRSS (▼), and SRSS (S) are shown above the bars.

ourselves to measuring the short-term proliferation after initial Ag and IL-2 contact.

Therefore, short-term CTL lines stimulated by M1(58-66) were established from the same donor but using PBMC collected 11 mo after those used in the previous studies. The cultures were sampled at 0, 2, 4, 6, and 7 days. The in vitro response of the T cells expressing those clonotypes was measured using the same clonotyping procedure as described for the precursor analysis.

The kinetic responses of seven BV17-J2.7<sup>+</sup> clonotypes whose initial precursor frequencies had been studied are presented in Figure 6. Overall, most clonotypes maintained their RF and showed only a slight increase in frequency. Clonotypes 10 and 12 showed an increase in RF (2.7- and 2.6-fold, respectively) over time, while clonotype 62 decreased slightly (1.2-fold). The increase in clonotype frequency for clonotypes 10 and 12 also fits with the data from the previous culture, wherein they were the two highest-frequency clonotypes after 5 wk of culture, but were lower in the PBMC (Figs. 3 and 5). It should be noted that while the changes in the RF of the clonotypes analyzed were not substantial, the overall RF of all BV17-BJ2.7<sup>+</sup> cells of this CDR3 size class increased during culture until it accounted for most of the surviving T cells.

#### *Precursor frequency of the high-frequency component over time*

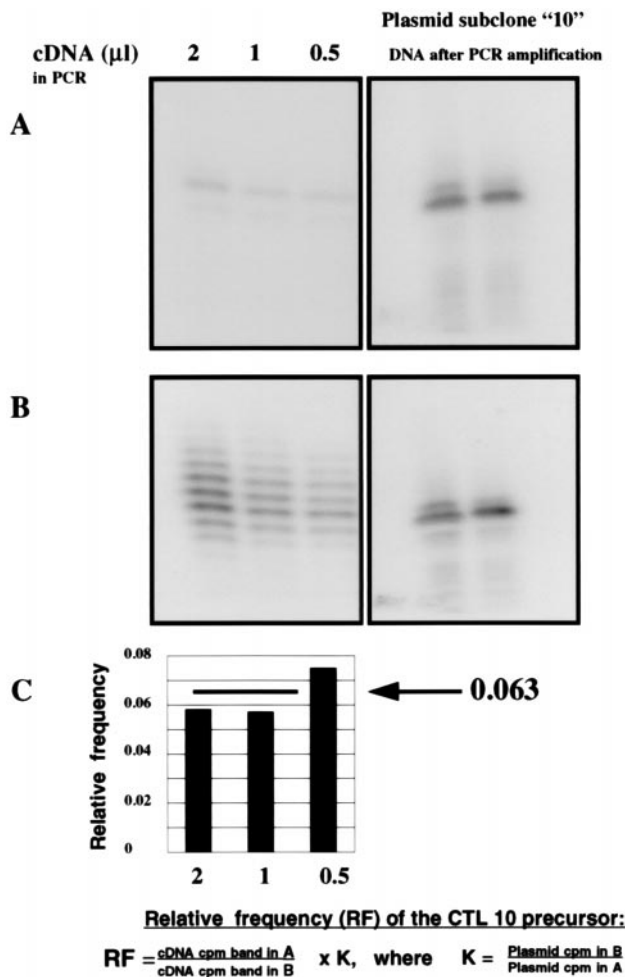
The previous section utilized PBMC collected 11 mo after the PBMC whose precursor frequency was analyzed in Figure 5. Day 0 of the kinetic analysis (Fig. 6) is in fact a precursor frequency analysis. Comparing the two sets of data showed that the high-frequency clonotypes were stable over at least 11 mo. This observation, together with the apparent lack of a high-proliferating com-

ponent to the repertoire, led to the hypothesis that these clonotypes were stable. The same seven clonotypes were subject to an additional precursor frequency analysis from PBMC collected 23 mo after the first sample. The result of this analysis together with the previous two analyses are shown in Figure 7. As expected, the relative frequencies of those clonotypes were stable over time. Despite some variation of the RF of clonotype 10, the general shape of the distribution is a stable phenomenon. Indeed, those clonotypes preserved their relative proportion, and their frequencies were stable in absolute values.

#### *Comparison of CDR3 and flanking sequence from clonotypes with similar recombination points does not support a somatic mutagenesis mechanism*

The possibility exists that the high frequency of very similar sequences we observe could have arisen by a somatic mutation process. To test this, we calculated the rate of divergence of the 14 bps that lie at the core of the CDR3 and compared this with the 7 bps of the flanking V and J regions. This was done in 59 of the J2.7-utilizing sequences that did not have what appears to be extensive deletion of the V or J. Assuming that the original rearrangement involved the generation of CDR3, which represents the consensus of these sequences, we calculate a 25.8% divergence from this consensus (213 of 826 bp) in the CDR core. The flanking 7 bp of V and of J region (14 bp total), which themselves should be equally likely targets for a mutagenesis mechanism, show 0.24% divergence (2 of 826 bp). In fact, outside the CDR3, there was only a 0.34% divergence (27 changes of 7920 bp), which can be explained by the error rate of reverse transcriptase and/or *Taq* DNA polymerase. Thus, we feel that there is no evidence for somatic





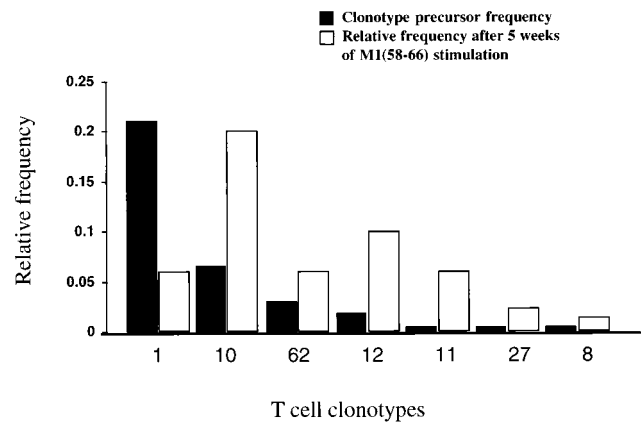
**FIGURE 4.** Evaluation of the clonotype precursor frequency of clonotype 10 in PBMC. cDNA dilutions were amplified using the BV17 primer and a labeled JB2.7 primer and subjected to electrophoresis. The volume of cDNA used in the PCR is shown above each lane (*left panels* in *A* and *B*). Duplicate dilutions of plasmid DNA containing the clonotype sequence were amplified as a control (*right panels*). *A*, DNA electrophoresed from the polyacrylamide gel was hybridized with the 10 cl-SSOP. *B*, Membrane from *A* rehybridized with BV17-SSOP. *C*, Clonotype RF calculated as described in *Materials and Methods*. The bars show the data for each lane and the arrow on the graph indicates on the average of the three cDNA dilutions used. The equation used is shown.

mutagenesis and that these sequences arose as a natural product of V-D-J joining followed by selection. These results are in keeping with the observation that the only report of somatic mutations in the TCR variable region occur in  $\alpha$ -chains but not in  $\beta$ -chains (19). Of course, the same logic can be applied to the possibility of “in vitro” mutagenesis caused by *Taq* polymerase or reverse transcriptase.

**Discussion**

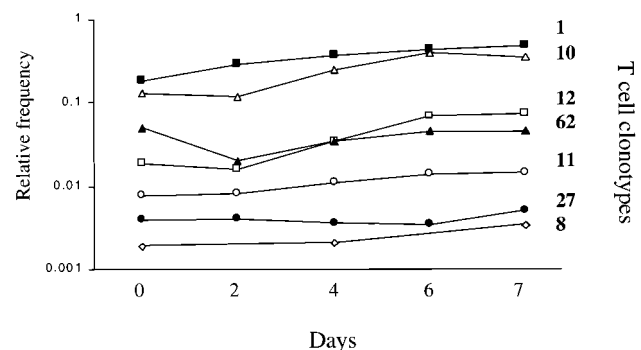
*Complexity of the responding repertoire*

Understanding the nature of the T cell repertoire generated during immune responses is of more than just academic value. The feasibility of immunotherapy and immunomodulation protocols will depend heavily on whether such responses are simple or complex. It is important to define characteristics of efficient repertoires. To date, most studies have described oligoclonal responses, based on high-proliferative capacity coupled to T cell avidity. Evidence for complex repertoires has been scant. However, most of the ap-

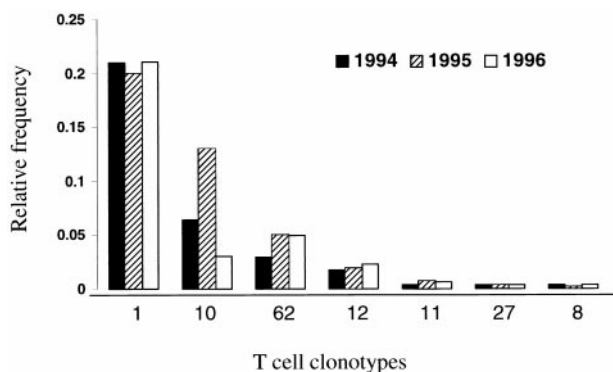


**FIGURE 5.** Relative frequencies for seven high-frequency clonotypes. Precursor frequencies were measured from PBMC (solid bar) and represent the average of the cDNA dilution series as shown by the arrow in Figure 4C. For comparison, the RF (colony number) from the analysis of the 5-wk cultures (open bars) is shown as the percent of total BJ2.7 colonies. Clonotypes are plotted in order of descending precursor frequencies and identified on the x-axis.

proaches used to date have relied on T cell cloning, which is not an optimal procedure for studying repertoire complexity. Two recent studies have used single cell PCR analysis of T cells classified as responders by flow cytometry based on surface markers (11, 12). These have shown that the naive and memory repertoires are oligoclonal. Our analysis is not based on any presuppositions about surface markers. We utilized a well-characterized recall Ag system to study CTL lines at the point at which they can be shown to display specific cytotoxicity. The lines displayed the expected characteristics of being predominantly BV17<sup>+</sup> and CD8<sup>+</sup>. Analysis of the BV17 TCR by cDNA cloning and sequencing identified 95 distinct clonotypes. These clonotypes all displayed CDR3 regions compatible with previously reported sequence motifs observed in T cell clones (13, 14). It has also been shown in the same Ag system that cytotoxic T cell lines treated with anti-BV17 mAb lose their ability to recognize target cells. The loss was proportional to the level of BV17<sup>+</sup> T cell depletion (14). While formally it is possible that only one or a few of the T cell clonotypes identified by their CDR3 sequence were responsible for the cytotoxicity, the previous work in this system (14) makes it more likely that most if not all were involved in the response. Even if some I-R-S clonotypes are not cytotoxic, this would pose the question of why clonotypes with these CDR3 characteristics were expanded in



**FIGURE 6.** RF of seven clonotypes sampled at different times during short-term culture. Relative frequencies (log scale) are shown for the clonotypes identified on the *right*.



**FIGURE 7.** Stability of clonotype precursor frequencies during a 23 mo time period. Precursor frequencies of the seven clonotypes were measured as shown in Figure 4 and described in *Materials and Methods*. Unstimulated PBMC collected in 1994 (solid bars), 11 mo later (1995, striped bars), and 23 mo later (1996, empty bars) were used.

the culture. We propose that this level of complexity provides insights into how T cell repertoires develop.

#### *Genesis of memory repertoires: proliferative mode*

In generation of the memory TCR repertoire, two major scenarios can be envisioned. In the first scenario, repertoire generation is based on proliferative capacity. Upon stimulation, the most avid T cell clones would rapidly expand and dominate the repertoire, whereas the least avid clones would decrease in RF in the repertoire. In this scenario, the repertoire would be characterized by a high-precursor frequency of one or a few clones that win the “proliferative race” after Ag engagement. The generation of class II-restricted T cell repertoires can take advantage of such a strategy, as has been shown for the mouse T cells specific to pigeon cytochrome *c* and restricted by I-E<sup>k</sup> (11).

Recent results with class I-restricted responses have also been interpreted in such a manner. Indeed, restimulation of mice with the Cw3 (170-179) peptide restricted by H-2K<sup>d</sup> in some cases led to the preferential expansion of fewer, more highly represented clonotypes (12). Implicit in such a mechanism is the focusing of memory repertoires upon restimulation, as highly proliferative clones would be expected to generate a larger percentage of the memory precursor pool after each Ag contact.

#### *Genesis of memory repertoires: progressive mode*

A second scenario would have a repertoire in which proliferation plays a less important role and no T cell clonotype comes to dominate the T cell repertoire. In this case, every T cell that becomes activated is approximately equal in its ability to proliferate in response to Ag. Thus, all the original responders should remain near their initial frequencies with respect to one another. Upon restimulation, the T cells would continue their limited expansion and perhaps be joined by newly arisen naive T cells that are stimulated for the first time. This process would lead to a high number of distinct clonal species without any single clonotype predominating. Such a strategy would be a “progressive” one with the repertoire expanding by a combination of polyclonal proliferation and new recruitment. Such a progressive strategy has not yet been described in either class I- or class II-restricted responses but is theoretically possible. We propose that our data provide evidence for such a mode of repertoire generation.

#### *The complexity of the anti-M1(58-86) response supports a progressive mode*

The complexity of the HLA-A2-restricted, anti-influenza A matrix peptide M1(58-66) cytotoxic response provides support for the existence of a progressive mode of repertoire generation. The 95 clonotypes identified directly here probably represent only a fraction of the total as our analysis was limited to 294 plasmid subclones that were generated from only a portion of the CTL lines. A statistical treatment of the data derived from each individual line is underway and indicates that the actual repertoire in the lines that we generated is being underestimated by at least twofold, with a majority of the new clonotypes representing the low-frequency tail of the distribution (E. Naumova et al., manuscript in preparation). It is possible that all 108 ways of encoding I-R-S may be encountered in a complete analysis of the repertoire.

The presence of clonotypes whose CDR3 differed by 1 bp raised the question about the possible role of somatic or in vitro mutagenesis in this process. Analysis of the regions flanking the CDR3 sequences indicated that somatic mutagenesis is unlikely and that in vitro errors have low probability, at the level of 0.24 to 0.35%. Thus, in a worse case scenario, the actual number of clonotypes we identified may be decreased from 95 to 94.

The polyclonality we report here is not a function of the molecular cloning and sequencing process because single stranded conformational polymorphism analysis of the initial RT-PCR product revealed a smear, indicating multiple sequences (data not shown). In contrast to the data reported here, an analysis of a DR1-restricted response to influenza M1(19–31) showed that most RT-PCR products were monoclonal, as evidenced by single strand conformational polymorphism and by colony filter hybridization. We have also used this methodology to analyze the complexity of the T cell response to a platelet allopeptide and have found apparent oligoclonal responses as evidenced by molecular cloning of the PCR band and colony filter hybridization with clonotype-specific probes (20).

#### *Lack of correlation of CDR3 sequence with clonal frequency or proliferative capacity is compatible with a progressive mode*

Further evidence for a progressive strategy came from our observations that circulating clonotype frequency could not be explained by in vitro proliferative capacity. The nucleotide and derived amino acid sequences of all the clonotypes from our experiments are grouped by their J gene and are shown in Table I. The <sup>1</sup>/<sub>S</sub>-R-S-<sup>A</sup>/<sub>S</sub> motif is present in almost all of the sequences. While the CDR3 nucleotide sequences are unique, many of the amino acid sequences are identical. Thus, when the J region is also the same, T cells expressing these TCRs would have identical β-chains despite the degeneracy of the nucleotide sequence in the CDR3 region. Comparison of clonotypes with identical CDR3 amino acid sequences indicated that the CDR3 sequence did not determine T cell clone (clonotype) precursor frequency (Fig. 3) or its ability to proliferate in vitro (Fig. 6).

Duplicate or triplicate analysis of the same time point showed that this method of measuring specific clonotypes is reproducible. Care was taken to insure that saturation of the PCR did not occur, as the most predominant clonotype could saturate at a cDNA concentration at which the other clonotypes were still responding in a concentration-dependent manner. A dilution series established a concentration at which the intensity of the most predominant clonotype was still a function of starting cDNA concentration.

It is still possible that there are other differences between clonotypes, i.e., the TCR  $\alpha$ -chain may change the avidity of the TCR-Ag-MHC interaction, and that this could explain some of the frequency and proliferation differences observed.

*Low proliferative capacity of the responding repertoire is compatible with a progressive mode*

Perhaps the strongest support for a progressive mode comes from the low proliferative capacity of the clonotypes involved. If the CDR3 sequences are used to identify particular clonotypes, there are a few clonotypes that proliferate more than others, but all of the measurable clonotypes proliferate modestly. This must be a function of the type of immune repertoire and not our culture or analysis conditions, because the same is not observed for a DR1-restricted response to influenza M1(19–31).<sup>6</sup> In this case, very high proliferation ( $\times 1000$ ) was measured within 7 days of culture.

If the T cells involved in an immune response do not undergo massive expansions, then the only way to generate a robust repertoire would be to progressively expand under repeated stimulation and to recruit new clonotypes when they were available. Thus, clonotypes that were recruited early or had a slight proliferative advantage (e.g., clonotypes 1, 10, and 12) will comprise the higher-frequency component, but their numbers would be augmented by many other clonotypes in the mid- and low-frequency range.

*Temporal stability of high-frequency components are compatible with early entry into the repertoire*

Under the progressive scenario described above, the earliest recruits would be slowly expected to comprise the highest-frequency component of the repertoire. To do this, they would have to be a stable population. We tested their stability by measuring the precursor frequency over 2 yr and found that the distribution of these clonotypes was stable with time. This is especially relevant for clonotypes that were poor in the proliferation assay (clonotypes 62, 27, and 8). Such stability would be compatible with a progressive mode of repertoire generation, whereas additions to the repertoire, and possibly some deletions, would take place in the low-frequency component. We are currently measuring of the low-frequency component in the 23-mo PBMC sample. Inherent in the progressive model is the need for restimulation to increase the frequency of the early recruits and those with a slightly increased proliferative capacity, and for new recruitment. While we assume that our test case has undergone such restimulation, one of the drawbacks of this analysis, and analyses in humans in general, is the lack of a well-controlled history of Ag exposure. The use of murine models will help in this regard. It is of some interest to note that the M1 peptide studied here does not vary from flu strain to strain, and the amino acid sequence is invariant in human isolates. This would increase the likelihood of restimulation of the same repertoire over repeated viral contact.

In the murine study most closely resembling ours, restimulation slightly decreased the number of countable clonotypes (12). However, there was evidence in all mice studied that new clonotypes had been recruited after the restimulation and that the higher-frequency component of the initial stimulation was generally maintained. These data would be compatible with our observations regarding the stability of the higher-frequency component and support a progressive mode of repertoire development. The apparent loss of the original low-frequency component could be due to

incomplete sampling favoring re-isolation of the higher-frequency clonotypes.

*Significance of the complex repertoires*

The delineation of an Ag-specific repertoire compatible with a progressive strategy, i.e., composed of multiple clonotypes with relatively similar rates of in vitro proliferation, has not yet been reported. This may be in part due to limited sampling of individual mice or humans in previous studies. In one recent study, a maximum of 13 clonotypes was reported from one of the mice analyzed. A Bayesian-based probability calculation indicated that the observed data were compatible with an average of  $\sim 20$  with an upper limit of 70 clonotypes (12). While the authors did not discuss the possibility of complex responses, our data and conclusions could be viewed as an extension of this previous observation. In the mouse from which 13 clonotypes were reported, the frequency distribution was similar to the one reported here. We propose that with a more extensive single cell PCR analysis, the low-frequency component (9 of 13 in mouse no. 1) would have increased. The paucity of previous reports of high polyclonality may be interpreted to indicate that it is infrequent and inconsequential. On the other hand, as more sensitive molecular genetic techniques are used to analyze repertoires thoroughly, such complex repertoires may prove to be commonplace. In addition to the mouse example cited above, the limited T cell cloning experiments performed to date with the Ag system used here (14) hint that such a complexity may be more widespread.

Why would two modes for generating memory TCR repertoire be necessary? The simplest hypothesis would be that repertoires in the CD4<sup>+</sup> and CD8<sup>+</sup> T cell lineages are generated differently. Indirect support comes from the much lower proliferative burst in class I-restricted responses (21), which would limit expansion. To generate a robust repertoire, the progressive strategy may be necessary in either lineage as long as the cell proliferation is limited or not directly linked to the avidity of the TCR-Ag-MHC interaction. The issue of whether complexity or concentration of the priming Ag can affect repertoire complexity (22) needs to be resolved. If a complex repertoire is required for efficient immunity, this may pose a limitation for simple immunotherapies or immunomodulatory regimens. However, it does offer the immune system multiple avenues to achieve effector function and allows subspecialization of different responding T cell lineages within the context of the response.

## Acknowledgments

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## References

- Zinkernagel, R. M., and P. C. Doherty. 1975. H-2 compatibility requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus: different cytotoxic T-cell specificities are associated with structures coded for in H-2K or H-2D. *J. Exp. Med.* 141:427.
- Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol.* 7:601.
- Heemels, M. T., and H. Ploegh. 1995. Generation, translocation, and presentation of MHC class I-restricted peptides. *Annu. Rev. Biochem.* 64:463.
- Madden, D. R. 1995. The three-dimensional structure of peptide-MHC complexes. *Annu. Rev. Immunol.* 13:587.
- Fields, B. A., E. L. Malchiodi, H. Li, X. Ysem, C. V. Stauffacher, P. M. Schlievert, K. Karjalainen, and R. A. Marriuzza. 1996. Crystal structure of a T cell antigen receptor  $\beta$ -chain complexed with a superantigen. *Nature* 384:188.
- Garcia, K. C., M. Degano, A. B. Stanfield, M. R. Jackson, P. A. Peterson, L. Teyton, and I. A. Wilson. 1996. An  $\alpha\beta$  T cell receptor structure at 2.5 Å and its orientation in the TCR-MHC complex. *Science* 274:209.

<sup>6</sup> Yassai M., Newton-Nash D., Naumov Y., and Gorski J., A requirement for IL-4 and IFN-gamma characterizes a portion of the T cell repertoire responding to a viral peptide in an in vitro recall response. *Submitted for publication.*

7. Garboczi, D. N., P. Ghosh, U. Utz, Q. R. Fan, W. W. Biddison, and D. C. Wiley. 1996. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature* 384:134.
8. Fink, P. J., L. A. Matis, D. L. McElligott, M. Bookman, and S. M. Hedrick. 1986. Correlations between T-cell specificity and the structure of the antigen receptor. *Nature* 321:219.
9. Winoto, A., J. L. Urban, N. C. Lan, J. Goverman, L. Hood, and D. Hansburg. 1986. Predominant use of a V alpha gene segment in mouse T-cell receptors for cytochrome C. *Nature* 324:679.
10. Taylor, P. M., J. Davey, K. Howland, J. B. Rothbard, and B. A. Askonas. 1987. Class I MHC molecules rather than other mouse genes dictate influenza epitope recognition by cytotoxic T cells. *Immunogenetics* 26:267.
11. McHeyzer-Williams, M. G., and M. M. Davis. 1995. Antigen-specific development of primary and memory T cells in vivo. *Science* 268:106.
12. Maryanski, J. L., C. V. Jongeneel, P. Bucher, J.-L. Casanova, and P. R. Walker. 1996. Single-cell PCR analysis of TCR repertoires selected by antigen in vivo: a high magnitude CD8 response is comprised of very few clones. *Immunity* 4:47.
13. Moss, P. A. H., R. J. Moots, W. M. C. Rosenberg, S. J. Rowland-Jones, H. C. Bodmer, A. J. McMichael, and J. I. Bell. 1991. Extensive conservation of alpha and beta chains of the human T-cell antigen receptor recognizing HLA-A2 and influenza A matrix peptide. *Proc. Natl. Acad. Sci. USA* 88:8987.
14. Lehner, P. J., E. C. Wang, P. A. Moss, S. Williams, K. Platt, S. M. Friedman, J. I. Bell, and L. K. Borysiewicz. 1995. Human HLA-A0201-restricted cytotoxic T lymphocyte recognition of influenza A is dominated by T cells bearing the V beta 17 gene segment. *J. Exp. Med.* 18:79.
15. Teng, J. M., and K. T. Hogan. 1994. Both major and minor peptide-binding pockets in HLA-A2 influence the presentation of influenza virus matrix peptide to cytotoxic T-lymphocytes. *Mol. Immunol.* 31:459.
16. Maslanka, K., T. Piatek, J. Gorski, M. Yassai, and J. Gorski. 1995. Molecular analysis of T-cell repertoires: spectratypes generated by multiplex polymerase chain reaction and evaluated by radioactivity or fluorescence. *Hum. Immunol.* 44:28.
17. Naumov, Y. N., E. N. Naumova, and J. Gorski. 1996. CD4<sup>+</sup> and CD8<sup>+</sup> circulating  $\alpha/\beta$  T-cell repertoires are equally complex and are characterized by different levels of steady-state TCR expression. *Hum. Immunol.* 48:52.
18. Yassai, M., E. Naumova, and J. Gorski. 1997. Generation of TCR spectratypes by multiplex PCR for T cell repertoire analysis. In *The Antigen T Cell Receptor: Selected Protocols and Applications*. J. R. Oksenberg, ed. Landes Company and Chapman & Hall, Austin, TX, p. 327.
19. Zheng, B., W. Xue, and G. Kelsoe. 1994. Locus-specific somatic hypermutation in germinal center T-cells. *Nature* 372:556.
20. Maslanka, K., M. Yassai, and J. Gorski. 1996. Molecular identification of T cells that respond in primary bulk culture to a peptide derived from a platelet glycoprotein implicated in neonatal alloimmune thrombocytopenia. *J. Clin. Invest.* 98:1802.
21. Hou, S., L. Hyland, K. W. Ryan, A. Portner, and P. C. Dougherty. 1994. Virus-specific CD8<sup>+</sup> T cell memory determined by clonal burst size. *Nature* 369:652.
22. Alexander-Miller, M. A., G. R. Leggatt, and J. A. Berzofsky. 1996. Selective expansion of high- or low-avidity cytotoxic T-lymphocytes and efficacy for adoptive immunotherapy. *Proc. Natl. Acad. Sci. USA* 93:4102.
23. Clothia, C., D. R. Boswell, and A. M. Lesk. 1988. The outline structure of the T cell alpha beta receptor. *EMBO J.* 17:3745.