Brief Definitive Report

Oligoclonal Expansion of Major Histocompatibility Complex Class I-restricted Cytolytic T Lymphocytes during a Primary Immune Response In Vivo: Direct Monitoring by Flow Cytometry and Polymerase Chain Reaction

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Summary

Previous T cell receptor (TCR) sequence analysis of a panel of 23 H-2Kd restricted cytotoxic T lymphocyte (CTL) clones recognizing the decapeptide HLA-CW3 170-179 revealed a striking conservation of TCR structure, in that all clones examined used Vβ10 and Jα pHDS58 segments. We show here that the primary response in vivo after intraperitoneal injection of DBA/2 mice with HLA-CW3 expressing transfectants of syngeneic P815 (H-2d) tumor cells is characterized by a dramatic expansion of CD8+ Vβ10+ CTL in the peritoneal cavity and draining (mesenteric) lymph node, as well as in peripheral blood. Additional analysis of TCR on HLA-CW3 immune populations by flow cytometry and polymerase chain reaction further indicates that the vast majority of responding CD8+ cells express restricted Vα domains, a dominant Jα segment (pHDS58), and a conserved CDR3 length for both α and β chains. This novel system provides a unique opportunity to directly monitor an oligoclonal primary antigen specific immune response in vivo at the single cell level independently of functional assays.

The TCR-α/β on CD4+ and CD8+ T cells recognizes processed peptides bound to MHC class II or I molecules, respectively. TCR repertoire diversity is determined by the number of distinct gene segments encoding variable (V, D, and J) elements of the α and β chains as well as by N-nucleotide additions that occur during somatic recombination. Although it is generally accepted that variable components of both TCR α and β chains contribute to the specificity of recognition of a given peptide-MHC complex, there are many documented cases in which preferences for certain TCR gene elements occur (1). A striking example of TCR preference concerns the recognition of the decapeptide HLA-CW3 170-179 in association with H-2Kd. In this system, 23 independent CD8+ CTL clones were all found to use the Vβ10 domain and a unique Jα segment (2). Furthermore, CD8+ peritoneal exudate lymphocytes (PEL) recovered from the site of secondary rejection of syngeneic tumor cell transfectants expressing HLA-CW3 were highly enriched in Vβ10+ cells (2). These findings prompted us to investigate whether the primary in vivo immune response to this peptide antigen could be followed directly by flow cytometric analysis of CD8+ Vβ10+ cells. We show here that CD8+ Vβ10+ cells increase dramatically in the lymphoid tissues and blood of DBA/2 mice during the course of a primary immune response to syngeneic P815 tumor cells transfected with the HLA-CW3 gene. This novel system provides a powerful tool to investigate many quantitative aspects of antigen-specific in vivo immune responses independently of functional assays.

Materials and Methods

Immunization. Adult female DBA/2 mice (Harlan Olac Ltd., Bicester, UK) were injected intraperitoneally with 10⁵ viable P815 tumor cells transfected with the HLA-CW3 gene (3). At various times thereafter, animals were bled and/or killed. PBL were purified by ficoll-hypaque centrifugation and PEL by nylon wool columns. Single cell suspensions of inguinal, axillary, or mesenteric lymph nodes and spleen were prepared by standard procedures.

mAbs and Flow Cytometry. The following mAbs were used: GK1.5 (anti-CD4); 53-6-7 (anti-CDS); B21.5 (anti-Vβ10; 4); B21.14 (anti-Vα8; 4); KTS0 (anti-Vα8; 5); and B20.1 (anti-Vα2; 6). For most experiments, triple staining was performed with FITC-conjugated anti-CD8, PE-conjugated anti-CD4, and biotinylated anti-Vβ10 (revealed with avidin-tricolor). The anti-Vα mAbs were revealed by FITC-conjugated goat anti-rat Ig (Caltag Laboratories, San Francisco, CA), and triple staining was completed with
PE-conjugated anti-CD8 and biotinylated anti-Vβ10 (plus avidin-tricolor). Samples were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA) equipped with LYSIS II software.

For cell sorting experiments, immune (day 12) PBL or PEL were double stained with FITC-conjugated anti-CD8 and biotinylated anti-Vβ10 (revealed with avidin-tricolor). CD8+ Vβ10+ and CD8+ Vβ10- cells were sorted on a FACStar PLUS® (Becton Dickinson & Co.) and tested for cytolytic activity after overnight incubation at 37°C (to allow reexpression of modulated TCR)

Cytolytic Assay. P815-CW3 transfectants and control P815 tumor cells were labeled with 51Cr as described (3). 2 × 10^5 51Cr-labeled target cells were mixed with varying numbers of sorted effector cells in V-bottomed microplates. 51Cr-release in supernatants was quantitated after 3.5 h.

cDNA-PCR and Direct Sequencing. RNA extraction, cDNA synthesis, and PCR using Vα8, Vβ10, Cα, and Cβ primers were carried out on CTL clones and freshly isolated PBL or PEL as previously described (2, 7). Direct sequencing of the PCR products was as described (8).

Results and Discussion

We have recently shown that a large panel of independent CD8+ CTL clones recognizing the peptide CW3 170-179 in association with H-2Kd utilize exclusively the TCR Vβ10 domain (2). This surprising result prompted us to examine the TCR Vβ usage of CD8+ T cells during a primary response to this antigen in vivo. For this purpose, DBA/2 (H-2d) mice were injected intraperitoneally with viable syngeneic P815 mastocytoma cells transfected with the HLA-CW3 gene. Previous studies have shown that such P815-CW3 transfectants are normally rejected by DBA/2 mice within 10-12 d and that CD8+ CTL clones specific for CW3 170-179 can be derived from such an immunization protocol (3, 9).

As shown in Fig. 1, PBL from DBA/2 mice taken 10 d after injection of 10^7 P815-CW3 transfectants contained a very high proportion (~65%) of CD8+ Vβ10+ cells, as compared with PBL from control mice (7% CD8+ Vβ10+). This enrichment of Vβ10+ cells occurred only in the CD8+ subset since CD4+ Vβ10+ PBL from immune mice remained at the same level (~10%) as in unimmunized controls. Analysis of various tissues at day 10 after immunization further indicated that CD8+ Vβ10+ cells were specifically elevated not only in blood but also in the peritoneal cavity, spleen, and draining (mesenteric) lymph node (Table 1). In contrast, no increase in CD8+ Vβ10+ cells was seen in distant (ei-
Figure 2. Kinetics and tissue distribution of CD8+ Vß10+ cells in P815-CW3 immune mice. Cells from the indicated tissues were triple stained as in Fig. 1. Data are plotted as percent Vß10+ cells (mean ± SD) gated on the CD8+ subset. (---) Mean of CD8+ Vß10+ cells in the corresponding tissue from control (uninjected) DBA/2 mice. No change in percent CD4+ Vß10+ cells was observed in any tissue or at any time (data not shown).

Figure 3. Cytolytic activity of HLA-CW3 immune (day 12) PBL (circles) and PEL (triangles) expressing Vß10. Sorted CD8+ Vß10+ (solid symbols) and CD8+ Vß10− (open symbols) cells were assayed for cytolytic activity on HLA-CW3 transfected P815 target cells at the indicated E/T ratios. No lysis of untransfected P815 target cells was observed (data not shown).

Figure 4. TCR Vα expression by CD8+ Vß10+ cells from P815-CW3 immune DBA/2 mice. PEL from day 12 immune mice were triple stained with mAbs against CD8, Vß10, and Vα8 or Vα2. Histograms represent Vα staining gated on CD8+ Vß10+ or CD8+ Vß10− populations. Similar results were obtained with two independent mAbs against Vα8 (KT50 or B21.14).
gene family and 4 of 23 clones used a particular Vα8 gene segment (p71). We therefore stained P815-CW3 immune PEL or PBL with mAbs B21.14 (4) or KT50 (5), both of which recognize Vα8-expressing T cells. Approximately 15% of CD8+ Vβ10+ cells from day 12 immune mice were stained with either of these mAbs, as compared with 3–4% in control DBA/2 mice (Fig. 4 and data not shown). In contrast, Vα2 expression was much lower among day 12 immune CD8+ Vβ10+ cells than in normal DBA/2 controls (Fig. 4 and data not shown). These data emphasize the restricted Vα repertoire of P815-CW3 reactive CD8+ T cells and again concur with previous estimates of Vα usage in our panel of CTL clones.

In addition to V domain preferences, other structural features of the TCR were highly conserved in our panel of HLA-CW3 specific CTL clones. In particular, all clones examined used the Jα pHDS58 segment and both α and β chains showed a highly conserved length of the CDR3 domain (2). To determine whether such features also applied to the TCR of CD8+ cells generated in a primary response in vivo to P815-CW3 transfectants, we performed direct sequencing of PCR amplified cDNA obtained from immune PBL and PEL populations.

To examine directly the β chain junctional region, a combination of Vβ10 and Cβ primers was used to amplify cDNA from day 10 PBL and PEL. The PCR products were directly sequenced with a second antisense Cβ primer located close to the VDJC junction (Fig. 5 A). As expected, the sequencing reaction was essentially unreadable through the junctional region (with the exception of two successive guanine nucleotides encoding a conserved glycine residue at position 97). However, the sequence of Vβ10 was clearly readable, indicating that most Vβ10-bearing cDNAs from the immune PBL or PEL populations had the same junctional length encoding six amino acids in the CDR3 loop. This nucleotide structure is identical to that found in β chains of CTL clones reactive with HLA-CW3 (2 and Fig. 5 A). The sequence of Vβ10-Cβ PCR products obtained from control unimmunized DBA/2 PBL was not readable in either the junctional or Vβ10 region (Fig. 5 A).

To analyze the TCR α chain structure of day 10 immune PBL or PEL, a combination of Vα8 and Cα primers was used. Sequencing of the PCR products was performed with a second Cα primer located close to the VJC junction (Fig. 5 B). Remarkably, the Jα pHDS58 sequence was clearly readable in the day 10 PEL and, to a lesser extent, PBL populations. Furthermore, after a short unreadable stretch corresponding to the VJ junction, the Vα8 sequence was also readable. These data demonstrate that most Vα8-bearing cDNAs in the immune PBL or PEL populations have a conserved CDR3 loop of nine amino acids incorporating the Jα pHDS58 sequence. Again, this nucleotide structure is identical to that found in Vα8-bearing HLA-CW3 specific CTL clones (2 and Fig. 5 B). No readable sequence was obtained when the same Vα8 and Cα primers were used to amplify cDNA for unimmunized DBA/2 PBL (Fig. 5 B). Taken together, the results indicate that the primary CD8+ T cell response to HLA-CW3 transfectants in vivo is highly oligo-clonal and that the TCR structure of most responding cells is identical to that described previously for established CTL clones with the same antigen specificity.

The observed increases in CD8+ Vβ10+ cells after immunization with P815-CW3 transfectants are even more striking when expressed in terms of absolute cell numbers. As illus-
Table 2. Increase in Absolute Numbers of CD8+ Vβ10+ Cells after P815-CW3 Immunization

<table>
<thead>
<tr>
<th>Mice</th>
<th>Cell source</th>
<th>Cells (× 10^6)</th>
<th>Percent CD8*</th>
<th>Percent Vβ10+ in CD8</th>
<th>Total CD8+ Vβ10+ (× 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>PBL</td>
<td>0.59 ± 0.07</td>
<td>5.8 ± 0.7</td>
<td>10.4 ± 2.1</td>
<td>0.0036 ± 0.0011</td>
</tr>
<tr>
<td>Immune</td>
<td>PBL</td>
<td>1.53 ± 0.20</td>
<td>31.1 ± 3.1</td>
<td>62.1 ± 4.2</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>Control</td>
<td>Spleen</td>
<td>87 ± 8</td>
<td>2.5 ± 0.4</td>
<td>9.0 ± 0.6</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Immune</td>
<td>Spleen</td>
<td>284 ± 44</td>
<td>5.3 ± 0.7</td>
<td>41.5 ± 3.2</td>
<td>6.3 ± 1.8</td>
</tr>
</tbody>
</table>

Data are mean ± SD of three individual control or day 10 immune DBA/2 mice. PBL values are calculated for 1 ml of whole blood (after Ficoll-Hypaque centrifugation).

The sustained increase in oligoclonal CD8+ Vβ10+ cells observed here during the primary response to HLA-CW3 transfectants is in marked contrast to recent studies of the T cell response in vivo to bacterial or retroviral superantigens. In the latter case, a limited proliferation of polyclonal Vβ-restricted superantigen-reactive T cells occurs, but this initial expansion phase is followed by the death of the majority of the reactive T cells in both CD4+ and CD8+ subsets (13-15). It will be of considerable interest to compare other physiological parameters of Vβ-restricted T cells involved in primary responses to peptide antigens and superantigens.

To our knowledge, the data documented in this report represent the first instance in which it has been possible to directly quantitate a primary peptide antigen specific T cell response in vivo at the single cell level independently of functional assays. This unique model system should allow a detailed analysis of the cellular requirements of such a response, as well as new insights into poorly understood areas such as T cell activation, recirculation, and memory.

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