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*J Immunol* 2004; 173:6627-6634; doi: 10.4049/jimmunol.173.11.6627

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Following Immunization Antigen Becomes Concentrated in a Limited Number of APCs Including B Cells

Craig A. Byersdorfer, Richard J. DiPaolo, Shirley J. Petzold, and Emil R. Unanue

Immunization with the hen egg-white lysozyme (HEL) protein induces T cells to various of its peptide determinants. The distribution of such T cells, however, does not correlate with the peptide level of each epitope on class II molecules. For this reason, we sought information on the cells responsible for Ag presentation following immunization, hoping to understand the lack of immunodominance in this system. By tracking HEL, and the ensuing peptide/MHC complexes, we find the following: 1) that HEL in the draining lymph node gets concentrated in a limited number of APC, particularly in dendritic cells and macrophages, 2) that these APC are functionally capable of presenting both major and minor determinants of HEL over a 100-fold range of Ag dose, and 3) that B cells present Ag gained at early times after immunization, but only following higher dose immunization. These data indicate that the breadth of a response is maintained over a wide dosage range by concentration of Ag in a limited number of cells presenting high levels and a great diversity of epitopes. *The Journal of Immunology*, 2004, 173: 6627–6634.

As CD4+ T cell responses require the recognition of specific peptide/MHC complexes displayed by APC, it is important to determine how Ag dose and levels of peptide presentation affect the ensuing T cell response. Recent studies from our laboratory gave two unexpected results when examining protein dose, display of hen egg-white lysozyme (HEL) peptides bound to I-A<sup>d</sup>, and the frequency/distribution of epitope-specific CD4<sup>+</sup> T cells following immunization. Based upon 60- to 300-fold display differences among HEL determinants (1), our expectation was that the highly represented major epitope (centered around the core peptide 52–60) would induce the predominant clonal response, while at limiting amounts of HEL the response to minor epitopes would not develop. To our surprise, clonal analysis of the HEL response indicated no direct relationship between levels of the four main peptide families and the relative distribution of CD4<sup>+</sup> T cell clones to all or any of them (2). Instead, frequencies of the responding T cells were proportional to the immunizing amounts of HEL over a 100-fold dose range, while the relative distribution to the various epitopes was unaffected. In other words, chemical dominance did not equate with immunological dominance when immunizing with HEL in CFA. Moreover, T cells recognized biochemically minor epitopes of HEL at doses of protein thought to be subimmunogenic.

Disparity between epitope display and total response has also been observed in the presentation of peptides by class I MHC molecules (3–6) (reviewed in Ref. 7). In at least two separate cases, following either viral inoculation with PR8 influenza virus or infection with the intracellular bacteria *Listeria monocytogenes*, the immunodominance hierarchy of the CD8<sup>+</sup> response did not correlate with predictions based upon the measured level of peptide presentation. Reasons cited for this apparent discrepancy included differences in MHC class I/peptide stability, lack of precursor T cells in the original repertoire, and immunomodulation by one CD8<sup>+</sup> T cell response against another.

In interpreting our results within the MHC class II system, one consideration is that adjuvant up-regulates costimulator molecules. This was tested using mice genetically deficient for B7-1 and -2 or CD40 (8). We found that the overall magnitude of the response was influenced by the B7-1, B7-2, and CD40 costimulatory molecules while the distribution of the response, either in the presence of absence of these costimulators, was equivalent, making the costimulatory effect independent of large differences in epitope display. Ab blocking of the negative regulatory molecule CTLA-4 gave similar results. Therefore, continued response against biochemically minor epitopes at lower doses and in the absence of costimulator molecules was difficult to explain unless the HEL was highly concentrated in a limited number of APC.

We also reasoned that the number or subset of APC capturing and presenting Ag might influence the response, with one subset responsible for priming to all epitopes of HEL, while another subset influences the total frequency of Ag-reactive cells. Ample evidence implicates dendritic cells (DCs) as important APCs in CD4<sup>+</sup> T cell priming, while previous studies have argued both for (9–17) and against (18–24) the role of B cells in this process. A scenario can be envisioned where, under conditions of adjuvant immunization, DCs are highly active in capturing and presenting the Ag, thereby initiating reactivity against many epitopes and ensuring the breadth of the response. Meanwhile, other resident lymph node cells, including B cells, might play an accessory role in expanding a response once initiated (12). This idea is supported by data from other laboratories demonstrating not only dynamic T cell interactions with DCs early and in distinct phases (reviewed in Refs. 25–28), but also delayed and subsequent migration of Ag-specific T cells to the edge of the B cell follicle (29).

To explain the disconnect in the CD4<sup>+</sup> system between T cell priming frequency and the relative distribution of the reactive T cells (under a multitude of conditions), studies were undertaken to quantitate the number and subset of Ag-bearing lymph node APC following immunization. We make the observation that 1) HEL gets highly concentrated in a few APC, mostly CD11b/c<sup>+</sup> but also...
CD19+; 2) CD11b/c+ cells are highly effective in functionally presenting both major and minor HEL epitopes over a variety of Ag concentrations, and 3) CD19− cells play an integral role in optimizing total anti-HEL T cell frequencies in a polyclonal response, are involved with presentation in a dose-dependent fashion, but are not necessary to ensure T cell distribution against all epitopes of HEL. This allows us to construct a model of how T cell frequency and distribution is affected by individual APC subsets following immunization.

Materials and Methods

Mice and reagents

B10.BR mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and 3A9 mice were a kind gift from the laboratory of Dr. M. Davis (Stanford University School of Medicine, Stanford, CA). μMT mice were obtained from The Jackson Laboratory and subsequently backcrossed six generations onto the B10.BR background. Mice were housed in microisolator cages in a specific pathogen-free facility and provided with food and water ad libitum, according to protocols approved by the Washington University Institutional Animal Care and Use Committee.

Immunization and cell handling

HEL and CFA were both obtained from Sigma-Aldrich (St. Louis, MO) and the HEL was further purified to eliminate contamination (1–2%) by non-HEL proteins. Mice were immunized with 10–0.1 nmol of unlabeled or 125I-labeled HEL (HEL+) in CFA distributed to both hind footpads (100 μl). At different time points following immunization, mice were euthanized and the popliteal lymph node lymphocytes harvested. A single cell suspension was generated by pressing lymph nodes through a 70 micron cell strainer.

Cell separation

Sets of APCs were separated based upon expression of: 1) CD11b and CD11c (CD11b/c−), containing DCs and macrophages; 2) CD11c alone (considered as DC and which may be CD11b+ or CD11b−); 3) CD11b depleted of CD11c (considered as macrophages, and termed CD11b+ for simplicity); and 4) CD19 (representing the B cell subset). Initial separations were done using magnetic bead sorting and the purity checked by flow cytometry with the designated Abs (CD11b-FITC, CD11c-PE, B220-FITC, and CD19-PE; all from BD Pharmingen (San Diego, CA)). Magnetic sorting was done as follows: in some experiments the cells bearing CD11b and CD11c were isolated together by incubating simultaneously with anti-CD11c and anti-CD11b beads (Miltenyi Biotec, Auburn CA); in other experiments, CD11c− cells were separated first by incubating with anti-CD11c beads, followed by subsequent isolation of the flow through using anti-CD11b beads. In either case, the flow through (lacking cells bearing CD11b/c−) was then used to separate out CD19+ cells using anti-CD19 beads (Miltenyi Biotec). Cells in the CD11b/c− subset were >70% CD19+, CD11c−, or CD11b/c− with a small contamination by B220+ cells (<10%).

Binding of radioactive Aw 3.18

A total of 0.5–2 × 10^6 cells in a total volume of 125 μl of DMEM containing 20% bovine calf serum and 0.02% sodium azide were incubated with 2.5 μg of [125I]-labeled Aw3.18 mAb for 2 h at 4°C. All cells were washed six times before transfer to microscope slides by cytocentrifugation and subsequent autoradiography. To determine background staining, a second set of cells was incubated as above but with a 50-fold excess of unlabelled Aw3.18.

Ag presentation assays and limiting dilution analysis

A total of 1 × 10^5 T hybridoma cells were added to a 96-well flat-bottom plate containing varying amounts of isolated APCs in a total volume of 200 μl of DMEM supplemented with 10% FCS. Following overnight incubation, 100 μl of supernatant was removed and tested for the presence of IL-2 using a standard CHLT response assay. The 3A9 hybridoma (31) was used to detect 48–62 complexes, while a new hybridoma was created to detect 20–35 complexes (LB11). To note, the response of the LB11 T cell hybridoma reflects the differences in display levels between the two epitopes (20–35 vs 48–62) and so in vitro requires 200-fold more total HEL protein than the 3A9 hybridoma to obtain a similar response. Limiting dilution analysis was performed as previously described (2) and was performed concurrently in both strains of mice. Frequency of HEL reactivity in the B10.BR animals ranged from 1/3,000 up to 1/9,000 over five separate experiments and were determined to be 1/29,300 and 1/19,200 in μMT mice for an average of 1/27,500.

Enrichment and CFSE labeling of 3A9 cells

Spleens, brachial, and cervical lymph nodes were removed from unmanipulated 3A9 mice, passed through a 70 micron strainer to generate a single cell suspension, red cells were lysed with ammonium chloride potassium lysis buffer (150 mM NH4CL, 10 mM KHCO3, 0.1 mM Na2EDTA-2H2O, pH 7.2–7.4), and the T cells were enriched by running over a CD3 enrichment column (R&D Systems, Minneapolis MN). Recovered cells were washed twice and labeled at a final concentration of 5 μM CFSE (Molecular Probes, Eugene OR) for 15 min at 37°C. Cells were washed extensively, resuspended in pyrogen-free saline and injected into mice at a concentration between 1.5 and 3.0 × 10^6 CD4+ 3A9 cells. Percentages were determined by staining with the clonotypic Ab 1G12, which recognizes the transgenic αβ TCR (32). Lymph nodes were removed 48 h postimmunization and stained with CD4-PE and 1G12-biotin, followed by streptavidin-allophycocyanin (BD Pharmingen). A histogram was drawn for cells that were gated 1G12, CD4 double positive. Average division and proportion of cells divided as previously described (2) and was performed on the recovered cells to determine the number of original undivided precursors, and then using this precursor number to calculate the average number of divisions for each cell.

Results

Radioactive detection of HEL and 48–62/I-Ak complexes

To identify APC that contained HEL, mice were immunized in the hind footpads with CFA and HEL labeled at high specific activity with 125I (HEL*). Mice were sacrificed at various times following immunization and the popliteal lymph node cells harvested and separated into various subsets using magnetic bead sorting. Most commonly CD11b+ and CD11c− cells were isolated together (CD11b/c−), followed by subsequent isolation of CD19+ cells. In other instances CD11c+ cells were isolated first (which could be either CD11b+ or CD11b−), followed by secondary isolation of the CD11b+ subset (always CD11c−). Total cellular radioactivity was measured before cells were placed on slides by cytocentrifugation and processed for autoradiography. In other experiments, cells were isolated from mice immunized with unlabeled HEL. In this case, harvested cells were incubated with 125I-labeled Aw3.18 Ab (Aw3.18*) to detect 48–62/I-Ak complexes, cytopsin onto slides, and the radiolabeled Ab was also detected by autoradiography.

Representative experiments are shown in Tables I, II, and III. The amount of HEL recovered at 24 h, regardless of the dose, was 1–2 femtomoles (fmol) per every nmol of HEL injected. We recovered an average of 3.3 × 10^3 HEL* CD11b/c− cells (with a range of 1.1–9.7 × 10^3) between two popliteal nodes. Most of the radioactive (96%) was found within these CD11b/c−
cells. For this subset, measurements before cytocentrifugation gave reliable cpm in the 4–6000 range. Total HEL measurements for the CD19+ population were unreliable, with cpm only 3-fold over background.

In terms of quantitation, the majority of cells at 24 h containing HEL+ were in the CD11b/c+ subset, averaging 1 cell per 100 cells isolated (Table IV). As regards timing, the frequency of HEL+ positive cells was lower at 8 vs 24 h (0.25% vs 0.71% in one representative experiment). Similar timing was observed in a parallel experiment assaying for peptide/MHC complexes by Aw3.18 reactivity. At later periods of time (12–96 h), the percentage of HEL+ cells did not vary and is represented in Table II at the 24 h time point. Between CD11c+ and CD11b+ subsets, the distribution of HEL+ cells was about equal (e.g., 1.3% vs 1.0%, respectively).

In all cases, the number of radioactive grains per cell in the CD11b/c+ group varied from 5 up to 100 per cell and positive cells had morphology typical of macrophages and DCs (Fig. 1A). Furthermore, the frequency of HEL+ bearing cells in the CD11b/c+ subset was about the same whether tested at 10, 1, and 0.1 nmol of HEL. In all analyses, cells were counted HEL+ positive if they clearly contained more radioactive grains than the surrounding background cells. In contrast to the CD11b/c+ subset, the frequency of HEL+ positive cells in the CD19+ subset was only 1 per 15,000 cells (0.008%). These positive cells were uniformly small lymphocytes (Fig. 1B) and often contained no more than 10 grains. (The number of grains per cell varied considerably within each set.) It is important to note that the time of exposure of the slides varied according to the dose of HEL that was examined: 4–7 days, 1–2 wk, or 7–10 wk for the 10, 1, and 0.1 nmol doses, respectively. Although not strictly quantitative, there were obvious differences in the content per cell for each of the three doses in both the CD11b/c+ and CD19+ sets.)

As regards 48–62/I-Ak complexes, Aw3.18+ cells (detecting 48–62/I-Ak) were reliably found in all subsets of cells at high doses of HEL. In the CD11b/c+, CD11b+, or CD11c+ subsets, the number of Aw3.18+ cells was about the same as the number of HEL+ positive cells (<2-fold higher), while in the CD19+ subset, the number of Aw3.18+ cells (relative to HEL+ bearing cells) was considerably higher. For CD19+ cells this went from <1/15,000 cells being HEL+ positive up to 1/2–400 cells being Aw3.18+ positive, an increase of nearly 50-fold. In toto, this data indicated that a small fraction of cells are HEL+ positive following immunization and that these subsets are also capable of displaying HEL’s major peptide determinant, 48–62, in complex with I-Ak.

**Presentation of HEL**

We next examined, by functional assays, which APC subsets contained MHC class II-bearing HEL peptides. Mice were immunized with unlabeled HEL at doses of 10 and 0.1 nmol and at 24 h the APC were isolated as described for the autoradiography experiments. Dose response assays were set-up with titrating amounts of APCs and T cell hybridomas recognizing either the chemically dominant 48–62 (3A9) or chemically minor 20–35 epitope (LB11).

Results from titrating APC assays can be seen in Fig. 2. At 10 nmol of HEL, both the CD11b/c+ and CD19+ subsets contained peptide/MHC and stimulated the indicator T cell hybridomas. For CD19+ cells, presentation ability at 10 nmol occurred over multiple experiments and was similar regardless of whether APCs were isolated by magnetic beads or flow-sorted (not shown). At the lower dose of 0.1 nmol HEL, only the CD11b/c+ cells presented (Fig. 2A, right panels). There was no presentation after immunizing with 0.1 nmol with up to 1 × 106 CD19+ APCs. In addition, while presentation from the CD11b/c+ cells changed only modestly with a 100-fold variance in Ag level (Fig. 2A, left vs right panels), presentation by CD19+ cells became an all or none response, being present at the higher dose and absent at the lower.

When lymph node cells were further separated into CD11c+ or CD11b+ subsets, peptide/MHC presentation to the 3A9 hybridoma was detected in both groups and at both high and low doses of HEL (Fig. 2B). However, CD11c+ cells were capable of engendering a 30-fold greater stimulation of the T cell hybridoma, as determined by comparing the half-maximal response of the two APC dose responses.

**Table I. Recovery of HEL per 10⁴ positive APCa**

<table>
<thead>
<tr>
<th>Cells</th>
<th>HEL Recovered (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b/c+</td>
<td>31.3 (n = 7)</td>
</tr>
<tr>
<td>CD11b+CD11c−</td>
<td>23.9 (n = 2)</td>
</tr>
<tr>
<td>CD11c+</td>
<td>32.9 (n = 2)</td>
</tr>
</tbody>
</table>

a B10.BR mice were immunized with 10 nmol of HEL+, the lymph nodes recovered at 24 h, and the cells sorted into the respective populations. Shown is the amount of HEL recovered in each cell population.

**Table II. Representative experimenta**

<table>
<thead>
<tr>
<th>Cells</th>
<th>% of Total LN Cells</th>
<th>Recovery HEL+</th>
<th>HEL (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>100</td>
<td>19.5 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>CD11b+</td>
<td>1.2</td>
<td>2.4 × 10⁵</td>
<td>3.5 × 10⁴</td>
</tr>
<tr>
<td>CD11c+</td>
<td>1.4</td>
<td>2.8 × 10⁵</td>
<td>6.2 × 10⁴</td>
</tr>
<tr>
<td>CD19+</td>
<td>9.2</td>
<td>1.8 × 10⁶</td>
<td>2.5 × 10⁴</td>
</tr>
</tbody>
</table>

a B10.BR mice were immunized with 10 nmol of HEL+, the lymph nodes recovered at 24 h, and the cells sorted into the respective populations. Data represent the average percentage of HEL+ cells or the total number (per mouse) of HEL+ cells recovered from two popliteal lymph nodes (total HEL = 22 fmol).

**Table III. Estimation of HEL+ cells per 10⁴ positive APCa**

<table>
<thead>
<tr>
<th>Calculation</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femtomoles per 10⁴ APC</td>
<td>31.3</td>
</tr>
<tr>
<td>Mean number HEL molecules per 10⁴ APC</td>
<td>1.88 × 10⁹</td>
</tr>
<tr>
<td>HEL molecules per single APC</td>
<td>1.88 × 10⁹</td>
</tr>
<tr>
<td>Estimated number of 48-62 complexes</td>
<td>1880</td>
</tr>
<tr>
<td>Estimated number of “minor” complexes</td>
<td>9</td>
</tr>
</tbody>
</table>

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<td>1880</td>
</tr>
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<td>9</td>
</tr>
</tbody>
</table>

**Table IV. Distribution of HEL+ and Aw3.18-positive APC (10 nmol at 24 h)b**

<table>
<thead>
<tr>
<th>Cells</th>
<th>HEL+ (%)</th>
<th>Aw3.18 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b/c+</td>
<td>1.0 (n = 9, 0.3–1.6)</td>
<td>3.1 (n = 2, 2–5)</td>
</tr>
<tr>
<td>CD11b+CD11c−</td>
<td>1.1 (n = 3, 0.9–1.5)</td>
<td>0.6 (n = 3, 0.3–0.9)</td>
</tr>
<tr>
<td>CD11c+</td>
<td>1.4 (n = 3, 0.8–2.2)</td>
<td>1.0 (n = 3, 0.7–1.5)</td>
</tr>
<tr>
<td>CD19+</td>
<td>0.008 (n = 3, 0.004–0.01)</td>
<td>0.22 (n = 3, 0.14–0.28)</td>
</tr>
</tbody>
</table>

b B10.BR mice were immunized with 10 nmol of radiolabeled or unlabeled HEL, the lymph nodes were recovered at 24 h, and the cells subsequently sorted. Numbers shown are percentage of total cells positive for either HEL+ or radiolabeled Aw3.18 Ab (detecting 48–62/I-Ak complexes). Numbers in parentheses represent the number of experiments followed by the range of percentages determined among all experiments. For Aw3.18 staining of CD11b/c+, CD11c+, or CD11b+ cells, the numbers represent the number of positive cells after subtracting the nonspecific background number (i.e., cells positive in the presence of 50-fold excess of unlabeled Aw3.18). For most experiments, the background number was 1 per 1000 cells and never exceeded more than 10% of the specific staining.
FIGURE 1. HEL* and Aw3.18* cells 24 h postimmunization. In A, mice were immunized with 10 nmol of HEL*, lymph nodes removed at 24 h, and cells sorted. Shown is an example from the CD11b/c* subset. In other experiments, B, mice were immunized with unlabeled HEL and 48–62/I-Ak complexes detected postsorting by incubation with radiolabeled mAb Aw3.18. A cell from the CD19* subset is shown by way of example. CD19* cells were uniformly small lymphocytes.

FIGURE 2. Functional presentation of peptide complexes from sorted APCs. A, Cells sorted from mice immunized with 10 or 0.1 nmol of HEL and then incubated with a 48–62 reactive (3A9, upper panels) or a 20–35 reactive (LB11, lower panels) T cell hybridoma to detect functional peptide/MHC complexes. In B, cells were sorted into CD11c* or CD11b* subsets and complexes detected with the 3A9 hybridoma (10 nmol, left panel; 0.1 nmol, right panel). Accumulation of HEL occurred as early as 8 h postimmunization, shown in C, where both the CD11b/c* and CD19* subsets give a similar 3A9 response at an immunizing dose of 10 nmol. In D, CD19* cells from CFA only or HEL-immunized lymph nodes were mixed with HEL-bearing µMT CD11b/c* cells, the CD19* cells sorted back out, and both groups tested for presentation to the 3A9 hybridoma. Lack of presentation by CD19* cells from the mice immunized only with CFA indicates no contamination by other HEL-bearing cells during the isolation process.
Last, we also wished to investigate how quickly various subsets gained access to the immunogen. For this, we immunized mice with 10 nmol of HEL in CFA, harvested the cells at 8 h postimmunization, and sorted them as previously. Results in Fig. 2C show that both the CD11b/c\(^+\) and the CD19\(^-\) subset presented peptide/MHC obtained by 8 h. In contrast to cells isolated at 24 h, however, both CD11b/c\(^+\) and CD19\(^-\) cells now present similar levels of the 48–62 epitope.

To exclude the possibility of contamination by non-B cells in our CD19\(^-\) cells, we performed the following manipulation. CD19\(^-\) cells from CFA only or 10 nmol HEL immunizations were mixed together with HEL-bearing \(\mu MT\) CD11b/c\(^+\) cells. After mixing, the CD19\(^-\) cells were sorted back out. Only CD19\(^-\) cells from lymph nodes originally immunized with HEL presented to the A3.A hybridoma (Fig. 2D), while CD19\(^-\) cells from a CFA-only immunization did not, despite mixing with and subsequent separation from HEL-bearing CD11b/c\(^+\) cells. This indicated that it is indeed CD19\(^-\) cells which are responsible for presentation in these separations, and not just a contamination artifact produced by HEL-bearing non-B cells.

**HEL response in B cell-deficient mice**

If B cell participation is physiologically relevant, lack of presentation should be detected in the immune response of \(\mu MT\) mice. Limiting dilution analysis was performed to compare the polyclonal T cell response between B10.\(\text{BR}\) and \(\mu MT\) strains. As is summarized in Table V, immunized B10.\(\text{BR}\) mice gave an anti-HEL T cell frequency of 1/5,000 total lymph node cells (calculated to be 1/550 CD4\(^+\) T cells), while in \(\mu MT\) animals this was reduced to 1/27,500 of total lymph node cells recovered (or 1/9,075 CD4\(^+\) T cells). Although the overall anti-HEL T cell frequency was 5-fold less in \(\mu MT\) animals, the 16-fold decrease in anti-HEL CD4\(^+\) T cell frequency was greater due to higher percentages of CD4\(^+\) cells in the lymph nodes of \(\mu MT\) mice. Despite this decrease in anti-HEL T cell frequencies, the relative distribution of T cells responding to the various epitopes did not vary between the two strains (Table VI).

To quantitatively examine T cell activation in vivo, we took advantage of CFSE labeling to track naive 3A9 T cells transferred with the 2 strains (Table VI).

### Table VI. Distribution of HEL-reactive T cells in B10.\(\text{BR}\) vs \(\mu MT\) mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency (of Total Cells)</th>
<th>% CD4(^+)</th>
<th>Frequency (of CD4(^+) Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.(\text{BR})</td>
<td>1/5000</td>
<td>11</td>
<td>1/550</td>
</tr>
<tr>
<td>(\mu MT)</td>
<td>1/27,500</td>
<td>33</td>
<td>1/9075</td>
</tr>
<tr>
<td>Difference</td>
<td>5.5</td>
<td>16.5</td>
<td></td>
</tr>
</tbody>
</table>

*a Cells were handled as in Table V. Shown is the distribution of reactivity in HEL-responsive T cells from either B10.\(\text{BR}\) or \(\mu MT\) animals. All numbers are a composite of five concurrent experiments for B10.\(\text{BR}\) and two experiments using \(\mu MT\) mice where >120 HEL-specific clones for B10.\(\text{BR}\) and 34 HEL-specific clones for \(\mu MT\) mice were tested for epitope specificity.

Discussion

These studies quantitated Ag presentation in a local lymph node, determining the number and sets of APC bearing HEL or peptide/MHC complexes at discrete windows of time. This quantitative information gives insights into the distribution of an immunogen among APC subsets and the role each may play in generating responsive T cells.

Based upon previous functional studies, we estimated that the number of cells bearing HEL in the first 24 h was limited, and yet must be extremely effective at priming T cells against a diversity of epitopes. This high concentration of HEL and/or peptide/MHC complexes in a small cohort of APCs helps explain priming of T cells against epitopes of HEL displayed in vastly different amounts. As the total amounts of HEL and the number of HEL-bearing cells is known, an estimate can be made on the average amount of HEL that each positive CD11b/c\(^+\) cell contained. In one experiment, shown in Table III, we recovered 31.3 fmol of HEL (1.88 \(\times\) 10\(^{10}\) molecules of HEL) in 1.5 \(\times\) 10\(^8\) total CD11b/c\(^+\) cells. Because only 0.67% of the CD11b/c\(^+\) cells were HEL\(^*\) positive (1 \(\times\) 10\(^4\)), this makes 1.88 \(\times\) 10\(^{10}\) molecules of HEL per \(\times\) 10\(^4\) CD11b/c\(^+\) cells, or 1.88 \(\times\) 10\(^6\) molecules per single APC. Using calculations from previous metabolic studies (30) where one 48–62 complex was generated for every 1000 molecules of HEL, 1.88 \(\times\) 10\(^6\) molecules yield 1880 complexes of the dominant 48–62 family of peptides (per APC) and \(\sim\)9 complexes of the minor epitopes (e.g., 18–35). At the lower dose, the figures come down proportionally to 188 complexes of 48–62 with one complex of the chemically minor epitopes. Were this same amount of HEL distributed in a larger number of APC, instead of the 1%, it could never explain the response to the minor epitopes, as further reduction would have resulted in the sole expression of the chemically dominant 48–62 peptides.

Of course, these figures of HEL\(^*\) may be an underestimate as HEL can be catabolized over time. Nevertheless, in the CD11b/c\(^+\) subset, estimates of Aw3.18\(^*\) cells are very similar to those with HEL\(^*\), indicating that such catabolism may not be a major factor. In CD19\(^-\) cells there is an appreciable difference in Aw3.18\(^*\) cells (vs HEL\(^*\)). In this case, the lower amount of HEL and higher levels of peptide/MHC may be a consequence of the few positive B cells having already catabolized the HEL to generate peptide/MHC complexes at the 24 h time point. Certainly, the functional presentation data agrees with B cells having access to the Ag at early time points and presenting peptide/MHC rapidly thereafter.

In terms of percentages, the number of HEL-positive CD11b/c\(^+\) cells was the same regardless of the dose used for immunization, but clearly the amount of HEL per positive cell varied. So while at

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**Table V. Frequency of HEL-reactive T cells in B10.\(\text{BR}\) vs \(\mu MT\) mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency of Total Cells</th>
<th>% CD4(^+)</th>
<th>Frequency of CD4(^+) Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.(\text{BR})</td>
<td>1/5000</td>
<td>11</td>
<td>1/550</td>
</tr>
<tr>
<td>(\mu MT)</td>
<td>1/27,500</td>
<td>33</td>
<td>1/9075</td>
</tr>
</tbody>
</table>

*a B10.\(\text{BR}\) or \(\mu MT\) mice were immunized with 10 nmol of HEL, the popliteal lymph nodes harvested day 7 postimmunization, and the cells placed in a limiting dilution analysis as described in Materials and Methods. The frequency of HEL-reactive cells was calculated assuming a Poisson distribution, and the frequency of reactive CD4\(^-\) cells was quantitated by multiplying the frequency of total cells by CD4\(^+\) percentages as determined by FACS analysis of the same population.
10 nmol there may be a higher percentage of cells with 100 grains, and at 0.1 nmol more cells with 20, the percentage of HEL*-positive cells did not vary with dose. The functional consequence of this lower level of HEL/APC may be the reduced presentation ability of CD11b/c⁺/H11001 at 0.1 vs 10 nmol of HEL (Fig. 2) and the reduction at 0.1 nmol of in vivo T cell priming (observed in the CFSE profiles of Fig. 3). The essential fact remains, however, that while the total amount of HEL per cell is lowered, the Ag continues to be highly concentrated in a limited number (1–2%) of CD11b/c⁺ APCs, which then maintain presentation of a diversity of epitopes.

We postulate that it is the dynamics of the in vivo process that explains how APC with so few peptide-MHC complexes are recognized so well and can generate such a robust immune response. Presentation in the local lymph node is a highly dynamic process with constant cellular traffic into and out of the node, and T cells and the APC interacting robustly over the course of several hours. Indeed, APC may continually come into or leave the node, die upon extended presentation, or be replaced by successive migration of new APC. Importantly, APC contacts with T cells may involve more than one cognate T cell interaction and/or last for extended periods of time. All these factors will, of course, result in changes in T cell recruitment and activation and are the current focus of a number of laboratories (33–40).

Involved in the dynamics of the response are also mechanisms that equalize the clonal response to both high and low abundance.
epitopes. To recapitulate, clonal equalization of the CD4+ anti-HEL response took place despite the absence of costimulatory molecules (8), at very low doses of HEL (2), and in the absence of B cells (shown here). Such an equal distribution could still be explained by: 1) a complex interplay between negative and positive costimulatory molecules not yet examined, 2) cooperativity rather than competition among activated T cell clones (41), and/or 3) differences in the primary T cell repertoire which are then borne out during the course of immunization.

In terms of subset-specific presentation, it is generally accepted that the DC is central in priming CD4+ T cells (42–44). The role of B cells, in contrast, has been more controversial. Our studies indicated that B cells capture HEL and effectively present its peptide determinants. This presentation by B cells was dose dependent, occurring only at higher doses of HEL, and with B cells acquiring the HEL as early as 8 h postimmunization. How the B cells acquire the HEL is not known, but could involve HEL that entered the node via lymphatics (45) and directly bound to B cells. Alternatively, the HEL could be cross-presented after an initial binding to DCs or macrophages. B cell uptake is not likely to be caused by a highly specific Ag receptor, as 1 in 455 B cells greatly overestimates previous calculations of any two B cells bearing the same high affinity specificity. This issue of specificity, however, remains to be directly examined in future studies.

Physiologically, the B cell is implicated in high dose presentation (and not low dose) by presence of anti-HEL serum Abs following immunization with 10 nmol, and a lack of anti-HEL Abs at lower doses (2). Moreover, CD4+ priming frequency in μMT animals dropped 16-fold under the same immunization regimen, lower doses (2), and in the absence of HEL response took place despite the absence of costimulatory molecules for lysozyme-specific T cell hybridomas. Immunity 12:83.

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References