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Characterization of Drug-Specific T Cells in Phenobarbital-Induced Eruption

Hideo Hashizume,1 Masahiro Takigawa, and Yoshiki Tokura

Phenobarbital has a high potential to elicit adverse reactions including severe skin eruptions and systemic involvements among the worldwide-prescribed drugs. Although phenobarbital hypersensitivity is thought to be mediated by T cells specific to the drug, its precise mechanism remains not fully elucidated. To characterize T cells reactive with phenobarbital, we generated drug-specific T cell clones and lines from PBMCs of patients with phenobarbital hypersensitivity showing various degrees of cutaneous and extracutaneous involvements. Although the TCR Vβ repertoire and phenotype in the T cell clones/T cell lines were heterogeneous among the patients, Vβ13.1+ and Vβ5.1+ clones or lines were raised from the individuals examined who possessed different HLA haplotypes. Histopathological examination suggested that Vβ5.1+CD8+ T cells and Vβ13.1+ T cells played a role in cutaneous and extracutaneous involvements, respectively. A Vβ13.1+CD4+ clone was found to proliferate in response to the Ag with processing-impaired, fixed APCs. Most of the clones and lines belonged to the Th2 phenotype, producing IL-4 and IL-5 but not IFN-γ upon phenobarbital stimulation. Clones/lines with Th1 or Th0 phenotypes also constituted minor populations. These observations clearly indicate the heterogeneity and a marked individual deviation of reactive T cell subsets among the patients in terms of CD4/8 phenotype, Vβ repertoire, Ag recognition pattern, and cytokine production; and thus provide evidence whereby each pathogenic T cell subset contributes to special elements of clinical presentation. The Journal of Immunology, 2002, 168: 5359–5368.

Anticonvulsants such as phenobarbital have a high risk in eliciting adverse hypersensitivity reactions characterized by not only cutaneous but also by extracutaneous involvements (1, 2). Toxic epidermal necrolysis (TEN) and Stevens-Johnson syndrome (SJS) are the most severe and acute life-threatening conditions with a high mortality rate. The patients with these conditions have severe malaise, high fever, and liver and renal dysfunction in addition to extensive epidermal bullae and epithelial detachment in cutaneous and mucous membranes (1, 3). The mild form of hypersensitivity reactions includes maculo-papular eruption (MPE) and bullous eruption with minimal systemic organ involvements.

Multiple organ responses in phenobarbital hypersensitivity are suggested to be immunologically mediated, but the precise mechanism remains unelucidated. T cells from the patients sensitized with anticonvulsants are activated in vivo and in vitro (4, 5). By immunohistochemistry, both CD4+ and CD8+ T cells infiltrate in the lesional skin (2, 6, 7). These data indicate that pathogenic T cells reactive with phenobarbital may induce the various aspects of clinical manifestations. One of the important issues is the immunological characterization of drug-specific T cells in relation to the organ-specific involvements. Recent findings on the role of cytokines, chemokines, and their receptors in migration of lymphoid cell subsets with affinity to a particular organ suggest that a variety of clinical manifestations as reflected by multiple organ involvements are attributed to T cell subsets expressing different organ-specific homing molecules (8). Thus, it is possible that cutaneous lymphocyte Ag (CLA)+ and αEβ7+ T cells induce skin damage and mucous membrane injuries, respectively (9). Another concern is the mechanism in which the multiple subsets of pathogenic T cells with different immunological characteristics are generated in response to phenobarbital. The antigenic determinants generated by the drug and its metabolites seem to be taken up by APCs in at least two different ways and variously recognized by T cells. One way is processing of the drug-related Ag-protein complex by APC followed by the presentation of antigenic moieties in the context of MHC. The other is the presentation of drug without processing by APC as observed in sulfamethoxazole and lidocaine hypersensitivity (10). The generation of multiple T cell subsets may underlie these various recognition profiles.

In the present study, we compared immunological characteristics of T cells stimulated with phenobarbital for short-term and drug-specific T cell clones (TCC) in the hypersensitivity patients with different clinical involvements. Multiple subsets of drug-specific T cells with different surface Ag expression, TCR Vβ usage, and cytokine profiles resided in skin and peripheral blood of the patients. These data strongly suggest that multiple organ involvements are mediated by functionally different T cell populations in phenobarbital hypersensitivity.

Materials and Methods

Patients and PBMC

One patient with TEN (patient A), three with SJS (B–D), five with MPE (E–I), and one with multiple bullous eruption (J) were enrolled in this study (Table I). They had a history of skin rash developed within 2 mo after the phenobarbital treatment was started and cleared by the cessation of the drug. Lymphocyte stimulation test (LST) with phenobarbital showed high (stimulation index (SI): >1.8) proliferating activity in 7 of 10 patients, confirming phenobarbital hypersensitivity. In other patients, the diagnosis was made on the basis of clinical features and courses. The patients with MPE and bullous eruption had minimal, if any, systemic involvements. Five normal individuals for controls were also investigated (mean age 33;
male, 3; female, 2). All patients were informed about the purpose of this study and agreed to participate. PBMC were isolated from patients with a standard Ficoll-Hypaque method at least 2 wk after the drug allergy episodes. Patients with TEN and SJS were selected for generation of TCC. PBMC were taken several times from patient A during 2-year follow-up periods, and from patients B–D during 1-year follow-up periods.

**Reagents, culture medium, and mAbs**

Phenobarbital sodium (purity >98%) was purchased from WAKO (Tokyo, Japan). Culture medium was RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS or 5% autologous serum. The EBV-infected B95-8 cell line was added to prevent EBV-induced T cell growth. The EBV-infected B cells were used as APC in the following experiments. The cells were stored at −120°C until use. For fixation, cells in 0.5 ml medium without serum were mixed with 12.5 µl of 2% glutaraldehyde (final concentration 0.05%) for 30 s at room temperature (11). The reaction was stopped by adding 1 ml of 0.2 M l-glycine.

**Generation of phenobarbital-specific TCC and T cell lines (TCL)**

PBMC were stimulated with 50 and 100 µg/ml phenobarbital in culture medium at a cell density of 10⁶ cells/well in a 24-well culture plate (Corning Glass, Corning, NY) for 7 days. The maximum concentration of phenobarbital used in this study was 100 µg/ml, because the inhibitory effect on the PHA-stimulated lymphocytic proliferation was occasionally observed above this concentration. These cells from bulk culture were seeded at 0.3 cells/well with 5 × 10⁶ autologous mitomycin C-treated (50 µg/ml) PBMC that were pulsed with 100 µg/ml of phenobarbital in a final volume of 100 µl in a 96-U bottom culture plate. Growing T cells were expanded with autologous or allogeneic mitomycin C-treated PBMC as feeder cells added at a 2 wk interval. Clonality was confirmed by immunofluorescence analysis with fluorescence-conjugated anti-CD3, -CD4, and -CD8 mAbs and a battery of anti-TCR Vβ mAbs. TCL were defined as monoclonal and oligoclonal T cell populations, respectively. Thus, cell lines raised were maintained in medium supplemented with 10–15 U/ml rIL-2 (Takeda Pharmaceutical, Tokyo, Japan).

**In vitro stimulation with phenobarbital**

PBMC at 2 × 10⁵ cells/well were stimulated with phenobarbital at varying concentrations. Alternatively, TCL and TCL at 2 × 10⁶ cells/well were cultured with mitomycin C-treated syngeneic APC at a ratio of 10:1 in the presence of phenobarbital. The culture was maintained at a final volume of 100 µl in a 96-well flat-bottom culture plate (Corning) at 37°C in 5% CO₂.

### Table 1. Patient profiles

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age/Sex</th>
<th>Type of Eruption</th>
<th>Involvements</th>
<th>Histology of Skin Lesion</th>
<th>Interval Between Drug Intake and Eruption (days)</th>
<th>Fever (&gt;38°C)</th>
<th>Liver Enzymes</th>
<th>Blood Cell Counts</th>
<th>LST</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>21/M</td>
<td>TEN/yes</td>
<td></td>
<td>Epidermal cell necrosis</td>
<td>25</td>
<td>Yes</td>
<td>53/121</td>
<td>4.5/1.6/0.24</td>
<td>109</td>
</tr>
<tr>
<td>B</td>
<td>73/M</td>
<td>SJS/yes</td>
<td></td>
<td>Not done</td>
<td>13</td>
<td>Yes</td>
<td>26/54</td>
<td>4.6/0.18/0.08</td>
<td>40</td>
</tr>
<tr>
<td>C</td>
<td>51/M</td>
<td>SJS/yes</td>
<td></td>
<td>Lichenoid tissue reaction</td>
<td>14</td>
<td>Yes</td>
<td>53/52</td>
<td>11.3/1.3/0.34</td>
<td>83</td>
</tr>
<tr>
<td>D</td>
<td>24/M</td>
<td>SJS/yes</td>
<td></td>
<td>Lichenoid tissue reaction</td>
<td>23</td>
<td>Yes</td>
<td>26/38</td>
<td>4.6/0.4/0.37</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>2/M</td>
<td>MPE/edema/no</td>
<td>MPE/no</td>
<td>Not done</td>
<td>8</td>
<td>Yes</td>
<td>60/18</td>
<td>4.6/0.05/0.48</td>
<td>7</td>
</tr>
<tr>
<td>F</td>
<td>75/M</td>
<td>MPE/edema/no</td>
<td>MPE/no</td>
<td>Not done</td>
<td>No information</td>
<td>No</td>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
</tr>
<tr>
<td>G</td>
<td>34/F</td>
<td>MPE/no</td>
<td>MPE/no</td>
<td>Not done</td>
<td>No information</td>
<td>No</td>
<td>47/32</td>
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<td>Not done</td>
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<tr>
<td>H</td>
<td>74/F</td>
<td>MPE/no</td>
<td>MPE/no</td>
<td>Not done</td>
<td>No information</td>
<td>No</td>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
</tr>
<tr>
<td>I</td>
<td>51/F</td>
<td>MPE/edema/no</td>
<td>MPE/no</td>
<td>Focal epidermal and perivascular infiltration</td>
<td>14</td>
<td>No</td>
<td>32/30</td>
<td>6.4/0.4/0.19</td>
<td>1</td>
</tr>
<tr>
<td>J</td>
<td>68/M</td>
<td>Multiple blisters</td>
<td>Massive</td>
<td>Perivascular infiltration</td>
<td>92</td>
<td>No</td>
<td>30/22</td>
<td>8.2/0/7.0/3</td>
<td>11</td>
</tr>
</tbody>
</table>

---

* Presence or absence of mucosal involvements are indicated as yes or no, respectively.
* Main histological findings of skin lesions are shown.
* AST, aspartic acid aminotransferase; ALT, alanine aminotransferase; CRP, C-reactive protein. For liver enzymes, blood cell counts and CRP, standard values are given.
* LST by phenobarbital, which is in detail described in Materials and Methods. Numbers are SI.
* The diagnosis had been improved by making a better discrimination of phenobarbital and ruling out of the other cause of rash.
were cultured and processed for the cell-division analysis by FCM. min as described previously (12). After washing in cold medium, the cells were cocultured with mitomycin C-treated syngeneic APC at a ratio of 10:1 in the presence of phenobarbital at 100 μg/ml. Alternatively, TCC and TCL were cocultured with biotin-labeled anti-IL-4, -IL-5, -IL-10, or IFN-γ mAb for 72 h at 37° C for 10 min. To observe the indirect fluorescence signal, the labeled mAb was added to each well at optimal concentrations. After incubation for 2 h at room temperature, the plates were washed, dried, and incubated for 2 h with the avidin-streptavidin complex at a concentration of 1:1000. A diaminobenzidine solution (Sigma Fast 3,3'-diaminobenzidine tablet sets; Sigma-Aldrich, St. Louis, MO) was used as a chromogen for visualization of the reaction. The black or dark brown spots that indicated footprints of cytokine-producing cells were counted in a dissecting microscope.

### Flow cytometry (FCM) analysis

Cells were washed once with HBSS containing 1% BSA and 0.1% NaN₃, and incubated with a panel of mAbs. For examination of cell surface molecules including TCR Vβ, culture periods varied depending on the experimental conditions. Cells were then subjected to flow cytometric and cell division analysis as below.

**Flow cytometry (FCM) analysis**

Cells were washed once with HBSS containing 1% BSA and 0.1% NaN₃, and incubated with a panel of fluorescence-conjugated mAbs for 30 min at 4°C in the dark. After washing, the harvested cells were resuspended in HBSS and subjected to FCM. Flow cytometry (FCM) analysis was performed with hematoxylin. Substitution of the primary Ab with isotype-matched IgG and omission of the primary Ab served as controls.

### ELISPOT assay for cytokine production

Cells were assayed at 1 × 10⁴ cells/well in a 96-well filtration plate (Multiscreen; Millipore, Molsheim, France) precoated with purified anti-IL-4, -IL-5, -IL-10, or IFN-γ mAb for 72 h at 37°C in 5% CO₂ in air for 24 h. The maximum proliferative response was achieved at a concentration of either 50 or 100 μg/ml in other patients. Therefore, we used the drug at concentrations of 50 and in air. For LST, cells were cultured for 48 h and [³H]Tdr (0.6 μCi/well; Amersham, Arlington, IL) were added to the wells 12 h before harvest. The cells were collected on glass-fiber filters using a cell harvester (Cambridge Technology, Watertown, MA) and radioisotope uptake was measured in a liquid scintillation counter. SI was calculated as cpm with reagent/cpm without reagent. For examination of cell surface molecules including TCR Vβ, cells were collected on glass-fiber filters using a cell harvester (Cambridge Technology, Watertown, MA) and radioisotope uptake was measured in a liquid scintillation counter. SI was calculated as cpm with reagent/cpm without reagent. For examination of cell surface molecules including TCR Vβ, cells were collected on glass-fiber filters using a cell harvester (Cambridge Technology, Watertown, MA) and radioisotope uptake was measured in a liquid scintillation counter. SI was calculated as cpm with reagent/cpm without reagent. For examination of cell surface molecules including TCR Vβ, cells were collected on glass-fiber filters using a cell harvester (Cambridge Technology, Watertown, MA) and radioisotope uptake was measured in a liquid scintillation counter. SI was calculated as cpm with reagent/cpm without reagent. For examination of cell surface molecules including TCR Vβ, cells were collected on glass-fiber filters using a cell harvester (Cambridge Technology, Watertown, MA) and radioisotope uptake was measured in a liquid scintillation counter. SI was calculated as cpm with reagent/cpm without reagent. For examination of cell surface molecules including TCR Vβ, cells were collected on glass-fiber filters using a cell harvester (Cambridge Technology, Watertown, MA) and radioisotope uptake was measured in a liquid scintillation counter. SI was calculated as cpm with reagent/cpm without reagent.
100 µg/ml in the following experiments. The proliferative response was completely inhibited by anti-HLA-DR mAb (G46-6) that binds the α subunit of MHC class II (13), but not by anti-HLA-A, -B, -C mAb, suggesting that the main response was restricted to MHC class II, and thus the majority of reactive cells were CD4⁺ T cells in patient A.

We confirmed the proliferation of CD4⁺ T cells in two different studies in patient A. First, CFSE, which covalently binds to cellular proteins and allows direct measurement of the cell division over several cell generations, was used to monitor the cell division induced specifically by Ag (12). Fig. 1B showed representative results obtained from patient A. After a 72-h culture, CD3⁺ T cells contained low but definite percentages of dividing cells, as seen in the left peak, even in the absence of phenobarbital (Fig. 1B, top panel, filled histogram). However, when cultured with the drug, divided cells were increased in number (Fig. 1B, open histogram). The divided cells were restricted essentially to the CD4⁺ population (Fig. 1B, middle panel), whereas the CD8⁺ subset showed a minimal peak (Fig. 1B, bottom panel). Second, when PBMC deprived of CD8⁻ cells in patient A were cultured with phenobarbital for 14 days, >90% of the cells were CD25⁺. In contrast, only 17% of CD4-depleted, Ag-stimulated cells expressed CD25. Stimulation with phenobarbital induced the expressing CLA mainly on CD4⁺, but not on CD8⁺ cells in patient A (data not shown).

We monitored the phenotype of phenobarbital-reactive lymphocytes periodically in five patients (patients A, B, E, I, and J). In patients A, B, E, I, and J, CD4⁺ cells preferentially proliferated to expand such that the ratio of CD4 to CD8 was increased as the cell division was prolonged (Fig. 1C). In contrast, in patient J, CD8⁺ cells vigorously expanded. Preferential proliferating activity of the CD8⁻ subset and its MHC class I dependency in patient J were confirmed by CFSE analysis (data not shown). In patients A, E, I, and J, the percentage of CLA⁺ cells was significantly higher (6.2–27.4%) than phenobarbital-pulsed healthy controls or untreated patients’ PBMC (2.4 ± 1.4%) after a 7-day cultivation. The expression of CLA molecules was elevated in a culture time-dependent fashion in most cases.

The percentages of cells expressing activation Ag and early activation Ag were examined by enumerating CD25⁺ and CD69⁺ cells, respectively, in the drug-pulsed total lymphocytes. In patient A, the percentages of CD25⁺ cells were increased from 26% at day 7 to 50% at day 14 and 11.3% at day 28. In contrast, the percentages of CD69⁺ cells were decreased from 6% at day 7 to 11% at day 14. In patients B, C, and H, the similar expression pattern of early CD69⁺ and sequential CD25⁺ waves was found (data not shown), providing another supportive evidence for phenobarbital-induced activation of lymphocytes. These results demonstrated that a majority of phenobarbital-reactive T cells was CD4⁺ in patients with TEN, SJS, and MPE; and CD8⁺ in a patient with bullous eruption. In addition, the reactive T cells expressed CLA at high percentages.

Skewed usage of particular TCR Vβs in phenobarbital-specific T cells from patients with different MHC haplotypes.

We next investigated the TCR Vβ usage by T cells reactive with phenobarbital in seven patients (Fig. 2). We tentatively defined the “skewed” usage of TCR Vβ as the percentage of a particular Vβ was above mean percentage +3 SD of the corresponding Vβ in normal individuals. Although percentages of particular TCR Vβ-bearing cells in freshly isolated patients’ PBMC were generally comparable to those in normal individuals, a slight increase in number was noted in Vβ3⁺ and Vβ9⁺ cells of patient A and Vβ3⁺ cells of patients F and I. After a 7-day culture with phenobarbital, the TCR repertoire of reactive T cells tended to skew to several Vβs in all cases. Thus, proliferating T cells preferentially expressed Vβ3 and Vβ5.1 in five of seven patients (Vβ3 in A, C, E, H, and J; and Vβ5.1 in A, C, F, H, and J) (Fig. 2, bottom).
Interestingly, we observed vigorous expansion of \( V_{\beta} 5.1^{+} \) cells, especially in patients A and J, who suffered from blister formation. Such skewed usage in \( V_{\beta} 5 \) was not observed in patients’ PBMC after PHA stimulation or phenobarbital-stimulated normal PBMC.

The TCR \( V_{\beta} \) usage of phenobarbital-reactive T cells was further analyzed at the clonal level in four patients with TEN (A) and SJS (B–D) (Table III). Twenty-nine of established 68 TCCs/TCLs from the patients (A, 14 TCCs; B, 5 TCCs; C, 2 TCCs; D, 5 TCCs) were listed in the box. n.t., not tested.

Table III. Profiles of phenobarbital-specific TCC

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clones/Lines</th>
<th>( V_{\beta} ) Usage of Clones</th>
<th>CD4 or 8</th>
<th>CLA(^{c} )</th>
<th>Representative SI(^{d} )</th>
<th>Cytokine Profile(^{e} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A0</td>
<td>2</td>
<td>4</td>
<td>60</td>
<td>3.6</td>
<td>2</td>
</tr>
<tr>
<td>A1, A2</td>
<td>5.1(^{f} )</td>
<td>8</td>
<td>0</td>
<td>1.7</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>8</td>
<td>4</td>
<td>45</td>
<td>2.4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>13.1(^{f} )</td>
<td>4</td>
<td>27</td>
<td>31.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A6</td>
<td>13.6</td>
<td>8</td>
<td>0</td>
<td>1.5</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>A7, A8</td>
<td>17</td>
<td>4</td>
<td>0</td>
<td>3.6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A9, A10</td>
<td>18</td>
<td>4</td>
<td>71</td>
<td>1.6</td>
<td>0</td>
<td></td>
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<tr>
<td>A11, A12</td>
<td>20</td>
<td>4</td>
<td>14</td>
<td>1.9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A13</td>
<td>21.3</td>
<td>4</td>
<td>0</td>
<td>1.6</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>A14</td>
<td>22</td>
<td>4</td>
<td>64</td>
<td>1.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>B1</td>
<td>5.1</td>
<td>8</td>
<td>2</td>
<td>1.7</td>
<td>n.t.</td>
</tr>
<tr>
<td>B2</td>
<td>13.1</td>
<td>4</td>
<td>n.t.</td>
<td>1.8</td>
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<tr>
<td>B3, B4</td>
<td>14</td>
<td>4</td>
<td>n.t.</td>
<td>1.8</td>
<td>1.2</td>
<td></td>
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<tr>
<td>B5</td>
<td>17</td>
<td>4</td>
<td>0</td>
<td>2.4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B6, B7</td>
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<td>4</td>
<td>n.t.</td>
<td>1.5</td>
<td>2</td>
<td></td>
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<tr>
<td>C</td>
<td>C1</td>
<td>5.1</td>
<td>4</td>
<td>4</td>
<td>2.2</td>
<td>n.t.</td>
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<tr>
<td>C2</td>
<td>13.1</td>
<td>4</td>
<td>2</td>
<td>2.0</td>
<td>n.t.</td>
<td></td>
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<td>D</td>
<td>D14</td>
<td>2</td>
<td>8</td>
<td>16</td>
<td>1.5</td>
<td>n.t.</td>
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<td>D31</td>
<td>7</td>
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<tr>
<td>D13</td>
<td>9</td>
<td>8</td>
<td>1</td>
<td>1.5</td>
<td>n.t.</td>
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<td>0</td>
<td>1.7</td>
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<tr>
<td>D21</td>
<td>14</td>
<td>8</td>
<td>10</td>
<td>1.8</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>D5</td>
<td>2, 5.1, 13.6</td>
<td>4/8</td>
<td>7</td>
<td>1.9</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>D12</td>
<td>5.3, 13.6</td>
<td>4/8</td>
<td>0</td>
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<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>D20</td>
<td>2, 23</td>
<td>4</td>
<td>0</td>
<td>1.5</td>
<td>n.t.</td>
<td></td>
</tr>
</tbody>
</table>

\( ^{a} \) HLA haplotypes of the patients were as follows: A, A2/24, B13/52, Cw3, DR2/12; C, DRB1, 0803/0901, DRQ1, 0601/0303; and D, A2/11, B13/60, Cw3/7, DR4/12.

\( ^{b} \) D5, D12, and D20 showed oligoclonality by FCM analysis and defined as TCL. Monoclonality was confirmed in the other cell populations.

\( ^{c} \) Percentage of CLA \(^{10} \) cells in TCC/TCL, respectively. n.t., not tested.

\( ^{d} \) SI \(^{2} \) was designated as phenobarbital-specific proliferation and representative SI of clones/lines was indicated.

\( ^{e} \) 0, Th0; 1, Th1; 2, Th2; n.t., not tested.

\( ^{f} \) Underlines indicate the shared TCR\( V_{\beta} \) among different patients.
and 3 TCLs) were confirmed to be phenobarbital-specific by showing proliferation activity (SI, >1.5) following stimulation with phenobarbital. No clone was established without phenobarbital stimulation.

Whereas the usage of TCR Vβ by these clones was heterogeneous among the patients, we again found the preferential usage of particular Vβ, despite different MHC haplotypes among the patients. As underlined in the third column of Table III, Vβ13.1 was used by CD4+ TCCs in patients A–C, and by a CD8+ TCC in patient D. Vβ3.1 was used by CD8+ TCCs in patients A and B, by a CD4+ TCC in patient C, and a TCL in patient D. Less preferential usage was noted in Vβ17 and Vβ21.3 by CD4+ TCCs of patients A and B. These data suggested that at least two types of T cell recognition of antigenic determinants were critical in clinical manifestations in phenobarbital hypersensitivity. First, there seems to be promiscuous Ag recognition by CD4+ and CD8+ phenobarbital-reactive T cells since the antigenic epitopes are formed between the drug and the different classes and haplotypes of MHC (14). The second type of recognition may be specific for each individual characterized by the interaction between particular Vβs and unique MHC haplotypes. Therefore, cutaneous and extracutaneous manifestations in phenobarbital hypersensitivity can be explained in part by T cells with a variety of Vβs with the different recognition patterns.

**Phenotype and TCR Vβ analysis of lymphocytes infiltrating lesional skin**

Histologic examination of the acute skin lesions from seven patients (A–D and H–J) revealed the common features of lichenoid tissue reactions showing dermal perivascular lymphocytic infiltration and basal cell damage (6). The degrees of lymphocytic infiltration and epidermal damages seemed to be closely associated with clinical features, moderate lymphocytic infiltration, and massive epidermal necrosis in TEN (patient A) and bullous eruption (J); marked epidermal lymphocytic infiltration in SJS (B–D); and mild epidermal and moderate dermal lymphocytic infiltration in MPE (H and I).

In patients with TEN (A), SJS (D), MPE (H), and multiple bullous eruption (J), we investigated which T cell subset infiltrated in the lesional skin by immunohistochemical staining (Table IV). In all patients, although CD8+ cells outnumbered CD4+ cells (<20%) in the epidermis, more CD8+ cells were associated with more severe epidermal damage. In contrast, numbers of CD4+ and CD8+ cells were comparable around dermal vessels. We performed further analyses for the TCR repertoire of infiltrating lymphocytes in patients H and J (Table IV). In patients H and J, ≥10% of infiltrating cells were Vβ5.1+ and less numbers of cells expressing Vβ3 in the epidermis (Fig. 3, A–C). In the dermis, infiltrating cells in the vicinity of the vessels of the deeper dermis were positive for various Vβs in patient H. The TCR Vβ usage of dermal infiltrating cells were more limited in patient J. Again, Vβ5.1+ and Vβ3+ cells were the main populations in the dermal infiltrate.

![Image](http://www.jimmunol.org/Downloaded%20from%20http://www.jimmunol.org/)

**FIGURE 3.** Immunohistochemical analysis for phenotype and TCR Vβ usage of infiltrating cells in the skin lesions from patients H (A) and J (B and C). Arrows show positive staining. Note Vβ5.1+ cells were located in epidermis and upper reticular dermis in both cases. A, Vβ5.1 expression from patient H with MPE; B, Vβ3+; C, Vβ5.1+ from patient J with multiple blister formation (indicated as Blister).

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Table IV. **Immunophenotype and TCR Vβ usage of infiltrating cells in skin lesions**

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4</th>
<th>CD8</th>
<th>Vβ3</th>
<th>Vβ5.1</th>
<th>Vβ13.1</th>
<th>Vβ21.3</th>
</tr>
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<tbody>
<tr>
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<td>n.t.</td>
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<td>n.t.</td>
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<tr>
<td>D</td>
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<td>+</td>
<td>±</td>
<td>±</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>H</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>J</td>
<td>±</td>
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<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
</tr>
</tbody>
</table>

*CD4+ and CD8+ cells among the infiltrating cells in or close to epidermis and around dermal vessels were evaluated on a five-point scale as follows: –, none; ±, <20%; +, 20–50%; ++, 50–80%; ++++, >80% of the total number of infiltrating cells.

*The respective TCR Vβ-bearing cells among the infiltrating cells in or close to epidermis and around dermal vessels were evaluated on a four-point scale as follows: –, none; ±, <5%; +, 5–10%; ++, >10% of the total number of infiltrating cells. n.t., not tested.*
These findings were in accordance with the observation that PBMC from these patients preferentially expressed Vβ5.1 and Vβ3 upon phenobarbital stimulation. Accumulation of Vβ5.1+ cells in the skin lesions seemed to be a specific event because T cells resided in normal skin do not preferentially use this Vβ (15). Collectively, although heterogeneous T cell subsets infiltrated skin lesion, CD8+ and Vβ5.1+ cells might locate in and close to the epidermis in phenobarbital-induced eruption.

Processing was required to induce proliferation of CD4+Vβ2+ TCC, but not CD4+Vβ13.1+ TCC

It is suggested that “processing” or “covalent binding” of drug to MHC on APC is not necessary for T cell activation in sulfonamide and lidocaine hypersensitivities (10, 16). Therefore, we examined requirement of processing by APC in phenobarbital hypersensitivity in clones A0 and A5 from patient A. Both clones expressed CD3, CD4, HLA-DR, and CLA to a various degree (Fig. 4A) 72 h after drug stimulation. Vβ2+ A0 and Vβ13.1+ A5 clones proliferated vigorously in response to phenobarbital presented by unfixed APC (Fig. 4B). When stimulated with the drug in the presence of glutaraldehyde-fixed EBV-transformed autologous B cells, only A5 proliferated as vigorously as these stimulated with unfixed APC (Fig. 4B).

Because fixed EBV-transformed autologous B cells were capable of presenting superantigen toxic shock syndrome toxin-1, but not conventional Ag-purified protein derivative, to T cells in PBMC from healthy individuals to proliferate (data not shown), it was unlikely that the fixation was imperfect for inhibition of the processing or altered the expression/synthesis of surface protein including MHC.

**Th2 cytokine production by phenobarbital-stimulated PBMC and phenobarbital-specific TCC**

To clarify the involution of Th1/2 paradigm in phenobarbital hypersensitivity, the cytokine production of fresh PBMC from two patients and of phenobarbital-specific TCCs was examined by the ELISPOT assay. PBMC from patients A and G produced IL-4 and IL-5, but no IFN-γ, upon stimulation with phenobarbital compared with individuals without phenobarbital hypersensitivity (Fig. 5A). Data of the ELISPOT assay in TCCs were shown in Table III and in detail in Fig. 5B. Twelve of 16 clones (11 clones from patient A and 5 clones from patient B) preferentially produced IL-4 and/or IL-5, representing Th2 phenotype (Table III). In patient A, most of the clones produced IL-4/IL-5 upon stimulation, reflecting the cytokine production of fresh PBMC. For example, clones A5 that were negative for CD69 produced significant numbers of IL-4 and/or IL-5 spots only following phenobarbital stimulation (Fig. 5B). A0, A10, and A11 that continuously expressed CD69 produced both IL-4 and IFN-γ even without stimulation, indicative of Th0 phenotype, but shifted to the Th2 cytokine production upon stimulation. In contrast, in A9 and A14, the production of IL-4/IL-5 was unchanged or down-regulated, whereas that of IFN-γ was up-regulated by phenobarbital stimulation, showing a shift from Th2 to Th0 cytokine profile. Alternatively, only one clone, B3, seemed to belong to the Th1 subset because the production of IFN-γ, but not IL-4 or IL-5, was observed. These data

**FIGURE 4.** Phenotypes (A) and proliferation in response to unfixed and fixed APC plus phenobarbital (B) of A0 and A5 clones. A, The expression of TCC surface molecules at the start of culture was analyzed by FCM. Numbers indicate the percentage of positive cells in the total. B, Clones A5 and A0 were stimulated for 48 h with APC fixed (■) or nonfixed (□) with glutaraldehyde in the presence (100 μg/ml) or absence of phenobarbital. Each bar indicates [3H]Tdr incorporation (cpm), and vertical bars represent SD. *, p < 0.0012; **, p < 0.04; ***, p < 0.005.
again showed heterogeneity of the cytokine profile in phenobarbital-specific T cell clones despite the fact that fresh blood cells showed Th2 cytokine production.

**Discussion**

In the present study, we tried to elucidate the relationship between clinical manifestations and participation of T cells with the particular immune phenotype. An important issue to be addressed was how pathogenic T cells mediated phenobarbital hypersensitivity in the context of TCR Vβ usage, Ag recognition, and cytokine production.

When stimulated in vitro with phenobarbital, PBMC in four patients with TEN, SJS, and MPE responded to show preferential proliferation of CD4⁺ cells, whereas CD8⁺ cells tended to die out under prolonged cultivation. In contrast, in a patient (J) with bullous eruption, in which massive epidermal necrosis was found histologically, the growth potential of CD8⁺ cells seemed to be superior to CD4⁺ cells in long-term culture. By clonal level analysis, most of TCCs/TCLs established from patients with TEN and SJS (A–C) belonged to CD4⁺ subsets, and CD8⁺ TCCs/TCLs constituted small populations. In contrast, CD8⁺ TCCs/TCLs were also raised preferentially from a patient with SJS (D) in addition to small numbers of CD4⁺ TCC/TCL. Although we could not generate TCC/TCL from a patient (J) with severe epidermal damage, CD8⁺ cells expanded in vitro vigorously, following phenobarbital-stimulation. In drug hypersensitivity in Ag-specific TCC/TCL, CD4⁺ TCCs are preferentially generated (17), indicating the major role of this cell type in the pathogenesis. However, it is not excluded that the culture conditions in our studies favored CD4⁺ cell growth and that other cells participated in the reactions as well. In this context, the emergence of CD8⁺ clones in patients with the severe forms might strengthen their functional significance. Skin pathology common to our patients was epidermal injuries with basal cell damage and dermal perivascular lymphocytic infiltration, so-called lichenoid tissue reactions (18–20). In confirmation of the previous study in anticonvulsant hypersensitivity (6, 7), CD4⁺ and CD8⁺ T cell subsets seemed to elicit different pathological changes, because CD8⁺ cells located at and close to the epidermis and both CD4⁺ and CD8⁺ cells at the perivascular area of the dermis. Establishment of drug-specific CD8⁺ TCCs from patients sensitized with penicillin emphasizes an important role of cells with the cytotoxic phenotype in eliciting bullous eruptions (21). Furthermore, CD8⁺ cell infiltration was still predominant even at a resolving phase of the eruptions in patient A (data not shown). These results suggested that both CD4⁺ and CD8⁺ cells contributed to the pathogenesis, although the size of each fraction among the phenobarbital-reactive cells varied depending on individual patients and that the preferential location of CD8⁺ subsets was characteristic in phenobarbital-induced skin manifestations.

The present study showed that multiple T cell subsets with shared Vβ were generated from individuals of different haplotypes who showed essentially the same pathological findings upon phenobarbital stimulation. These included Vβ3⁺ and Vβ5.1⁺ T cells in short-term culture and Vβ5.1⁺, Vβ13.1⁺, Vβ22⁺, Vβ17⁺, and Vβ21.3⁺ cells in TCCs/TCLs. We could raise Vβ13.1⁺ clones but no Vβ3⁺ clones in TCC analysis; whereas Vβ3⁺ cells expanded prominently, but Vβ13.1⁺ cells did not outgrow in short-term culture. The change in growth potential during culture periods has been frequently observed (22) and might be due in part to the in vitro microenvironmental conditions in TCC generation. These data indicated that a variety of clinical manifestations in skin and other organs was mediated by multiple T cell subsets that respond to different antigenic determinants generated through catalysis of drug by hepatic and extrahepatic enzymes or via bindings of drug to the particular regions in cryptic self peptides of MHC class II molecules as observed in nickel (23) and penicillin (24). Of particular importance is the relationship between Vβ5.1⁺ and Vβ3⁺ T cells of CD4 and CD8 phenotypes and skin pathology. First, large numbers of Vβ5.1⁺ and Vβ3⁺ cells infiltrated in and close to the epidermis and at the dermal perivascular areas in MPE (patient H) and multiple bullous eruption (patient J). Second, short-term drug stimulation resulted in the appearance of these two subsets at high frequencies in five of seven patients (A, C, F, H, and J). Finally, phenobarbital-specific Vβ5.1⁺ TCCs/TCLs were generated from all the patients with TEN and SJS. Therefore, infiltration of the Vβ5.1⁺ and Vβ3⁺ subsets might be relevant to the various types of cutaneous manifestations, and Vβ5.1⁺ CD8⁺ cells, in particular, to epidermal damage. The use of certain TCR Vβ by phenobarbital-specific T cells was not very convincing as the oligoclonality of the drug-specific clones seemed to be less stringent in our data. The clonal-level analysis suggested that Vβ13.1⁺ cells, although not found in the lesional infiltrate, contributed to the extracutaneous manifestations.

Traffic of pathogenic T cells by means of some essential molecules such as CLA (2), cytokine receptors, and chemokine...
The interaction between T cells specific for lidocaine, sulfamethoxazole, and amoxicillin and APC (16, 27). Direct binding of nickel to particular amino acids of cryptic MHC peptides results in conformational change of the MHC-peptide-metal complex so as to be recognized by nickel-specific T cells irrespective of APC alleles (28). Furthermore, a monomorphic surface molecule, CD39, can function as additional recognition structures on haptenated target cells for HLA-A1-restricted hapten-specific CD8^+ T cells (29). Therefore, the establishment of T cell clones with shared Vβs at high frequencies from different patients might reflect promiscuous recognition of phenobarbital presented by APC in various ways.

As we addressed herein, phenobarbital-related Ag recognition without processing by APC took place in special T cell subsets responsible for the hypersensitivity. For example, CD4^+ Vβ13.1^+ TCC from patient A proliferated with processing-impaired APC in the presence of phenobarbital. Proliferation of this clone was inhibited by addition of anti-DR mAb, suggesting that the Ags would directly bind to the α-chain of HLA-DR molecule (13). This type of Ag recognition is observed in sulfamethoxazole and lidocaine hypersensitivity in which the drugs become immunogenic by simply interacting with the MHC-peptide complex (30). Another concern is cross-reactivity of drug-reactive T cells to multiple neoantigenic determinants. For example, T cells respond polyclonally to several antibiotics including penicillin G in patients sensitized with β-lactams (31). The CDR3 region of TCR in β-lactam-reactive TCC established from these patients has no common sequence that can recognize the penicilloyl group, indicating the generation of cross-reactive Vβs upon drug stimulation. This possibility is also likely in phenobarbital hypersensitivity.

Fresh PBMC from two patients and the majority of established TCLs may re-reflect promiscuous recognition of phenobarbital pre-TCCs upon stimulation were various, that is, enhanced production of cytokines or chemokine receptors in phenobarbital-reactive cells. Alternatively, it was possible that some of these populations attacked organs other than skin by expressing different organ-specific adhesion molecules and chemokine receptors on their cell surface, resulting in liver dysfunction and blood abnormalities. For example, pathogenetic roles of Vβ13.1^+ clones were strongly suggested by their establishment at high frequencies from different patients. However, virtual absence of T cells with this phenotype among the infiltrate in skin lesions indicated that Vβ13.1^+ cells might have had little potential of skin-homing.

T cell recognition of antigenic moieties on APC is not so stringent as has been suggested, and promiscuous under certain settings. Allele-unrestricted presentation of haptenic small compounds by MHC class II molecules is recently described in the interactions between T cells specific for lidocaine, sulfamethoxazole, and amoxicillin and APC (16, 27). Direct binding of nickel to particular amino acids of cryptic MHC peptides results in conformational change of the MHC-peptide-metal complex so as to be recognized by nickel-specific T cells irrespective of APC alleles (28). Furthermore, a monomorphic surface molecule, CD39, can function as additional recognition structures on haptenated target cells for HLA-A1-restricted hapten-specific CD8^+ T cells (29). Therefore, the establishment of T cell clones with shared Vβs at high frequencies from different patients might reflect promiscuous recognition of phenobarbital presented by APC in various ways.

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Fresh PBMC from two patients and the majority of established clones from three individuals produced IL-4/5 and IL-10, but not IFN-γ in response to phenobarbital. This may explain blood eosinophilia, a hallmark of Th2 cell activation, observed in several patients. Furthermore, the cytokine profiles of other drug-specific TCCs upon stimulation were various, that is, enhanced production of both Th1 and Th2 cytokines, and shifts from Th0 to Th2 and from Th2 to Th0 cytokine production. A previous report has suggested that in vivo polarized expression of cytokines are dependent on the chronology of the clinical reaction (32). Although we could not confirm this finding, the pattern of cytokine profiles in TCCs/TCLs may figure the variation of clinical manifestations in phenobarbital hypersensitivity.

The present data suggests that a spectrum of the clinical presentations ranging from severe forms such as TEN and SJS to mild ones with minimum systemic involvements in phenobarbital hypersensitivity can be explained by pathogenic T cells with different immune characteristics. The drug-reactive T cells may arise through presentation of a variety of antigenic determinants derived from phenobarbital and its metabolites by APC with and without Ag processing. These T cell subsets bearing different TCR Vβs are likely to express homing receptors or chemokine receptors for either skin or other organs and produce Th1 and/or Th2 cytokines at the migrated organs. Future challenges will include identification of antigenic determinants specific for each clinical manifestation and elucidation of the molecular mechanism in the interaction between these determinants and pathogenic T cells.

References


