INTRATHYMIC ELIMINATION OF Mls-a-REACTIVE (Vβ6+ )
CELLS DURING NEONATAL TOLERANCE INDUCTION TO
Mls-a-ENCODED ANTIGENS

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The mechanism(s) underlying the development and maintenance of T cell tolerance to self antigens is not clear, but is generally believed to involve either deletion, “anergy,” or suppression of self-reactive clones (1, 2). Experimental attempts to discriminate between these three possibilities have been severely limited by the necessity of using complex functional assays to assess the presence (or absence) of self-reactive clones. Whereas limiting dilution microassays present clear theoretical advantages over conventional assays, analysis of the tolerant state using this technology has thus far led to controversial results (2). Furthermore, limiting dilution assays cannot distinguish between the clonal deletion and “anergy” models.

A more direct approach to assess the cellular basis of tolerance induction is suggested by recent data (3, 4) indicating that T cell receptor (TCR) Vβ domain usage can in certain cases be strongly correlated with reactivity to specific antigens. By exploiting such correlations, it has been possible to demonstrate that tolerance to constitutively expressed antigens such as those encoded by MHC class I (5) and Mls-a (4) genes is mediated via elimination of self-reactive clones. In the present article we have extended this approach to investigate the basis of experimentally induced neonatal tolerance. Our data indicate that neonatally induced tolerance to Mls-a antigens is likewise accompanied by the intrathymic elimination of Mls-a-reactive cells, thus supporting the clonal deletion model.

Materials and Methods

Mice. C57BL/6, BALB/c, and DBA/2 mice were obtained from the animal facility of the Swiss Institute for Experimental Cancer Research, Épalinges, Switzerland.

Cell Preparation. Lymph node (inguinal + axillary) or spleen cell suspensions were prepared by homogenization in a loose-fitting tissue homogenizer. Cortisone-resistant thymocytes (CRT) were obtained after two intraperitoneal injections of hydrocortisone acetate (4 mg/mouse), 24 h and 48 h before mice were killed.

Tolerance Induction. BALB/c mice were injected intraperitoneally with 10⁶ viable nucleated DBA/2 (or control BALB/c) spleen cells within 24 h of birth.

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Monoclonal Antibodies. mAbs directed against Lyt-1 (53-7.3) and Lyt-1.1 (7.20-6.3) were provided by Drs. J. Ledbetter and J. Klein, respectively. mAb KJ16-133 (6) reacts with mouse TCR using products of the Vp 8 gene family. mAb 44-22-1 was originally derived against a H-2D<sup>b</sup>-specific cytolytic T cell clone (7). We have recently shown (Hengartner, H., et al., manuscript in preparation) that this mAb reacts with all TCR using V<sub>pβ</sub>. Cell Culture. In some experiments, CRT from control or tolerant BALB/c mice (4 x 10<sup>5</sup> in 2 ml) were cultured for 5 d with PMA (1 ng/ml), ionomycin (0.25 ug/ml), and human rIL-2 (200 U/ml). Recovered cells were analyzed for expression of V<sub>pα</sub> and V<sub>pβ</sub> as described below.

Flow Microfluorometry (FMF). Lymph node cells or CRT (5 x 10<sup>5</sup> in 100 μl) were stained sequentially with mAbs (undiluted hybridoma culture supernatant) followed by fluoresceinated goat anti-rat or goat anti-mouse Ig (Tago Inc., Burlingame, CA). Samples were passed on a FACS II flow cytometer (B/D FACS Systems, Sunnyvale, CA) gated to exclude nonviable cells. Fluorescence histograms (representing 10<sup>4</sup> viable cells) were accumulated on a logarithmic scale. Data are presented as percent positive cells after subtraction of samples stained with the fluorescent conjugate alone.

IL-2 Production and Assay. As described in detail elsewhere (8), lymph node cells from control or tolerant BALB/c mice were depleted of CD8<sup>+</sup> cells by treatment with rat IgM anti-Lyt-2 mAb 3.168.1 plus complement. Recovered cells (5 x 10<sup>5</sup>) were cultured with 5 x 10<sup>5</sup> T cell-depleted BALB/c, DBA/2 or C57BL/6 spleen cells in 200 μl in flat-bottomed microcups. After 48 h, supernatants were harvested and titrated for IL-2 activity on IL-2-dependent CTLL cells. Data are expressed as IL-2 U/ml (1 U is equivalent to 0.3 ng human rIL-2).

Results and Discussion

As expected from previous studies (9), lymph node cells from adult (8 wk) BALB/c (H-2<sup>d</sup>, Mls<sup>b</sup>) mice injected neonatally with DBA/2 (H-2<sup>d</sup>, Mls<sup>+</sup>) spleen cells were tolerant to Mls<sup>b</sup> antigens, as indicated by their inability to secrete IL-2 in response to T cell-depleted DBA/2 spleen cells (Fig. 1). This tolerance was specific, in that the neonatally injected mice responded normally to third-party C57BL/6 (H-2<sup>b</sup>, Mls<sup>+</sup>) spleen cells.

To investigate the cellular basis of this induced nonresponsive state, we took advantage of our recent observation (4) that T cell reactivity to Mls<sup>b</sup> antigens in vitro is strongly correlated with expression of TCR using the product of the V<sub>pβ</sub> gene. Such V<sub>pβ</sub>-bearing receptors can be readily quantitated by their specific reactivity.
with mAb 44-22-1 (Hengartner, H., et al., manuscript in preparation). As shown in Fig. 2 and Table I, ~10% of lymph node cells in normal BALB/c mice were stained by the Vp6-specific mAb 44-22-1, whereas ~20% reacted with the control Vp8-specific mAb KJ16-133. In the lymph nodes of functionally tolerant mice, expression of Vp6 was dramatically reduced (0-3.4%), whereas expression of Vp8 remained at virtually normal levels (14-18%). This phenomenon was not restricted to mice of the H-2\textsuperscript{b} haplotype, since C3H/He (H-2\textsuperscript{k}, Mls\textsuperscript{a}) mice rendered neonatally tolerant to AKR/J (H-2\textsuperscript{k}, Mls\textsuperscript{a}) spleen cells likewise had very few Vp6\textsuperscript{+} T cells (0.5-1.2%) as compared with untreated controls (10-12% Vp6\textsuperscript{+}; see reference 4).

Taken together, these data demonstrate that TCR using Vp6 are selectively deficient among peripheral T cells of mice rendered neonatally tolerant to Mls\textsuperscript{a} antigens.

The thymus is generally accepted to be the anatomical site of T cell tolerance induction (1, 2). To determine whether the elimination of Vp6\textsuperscript{+} cells in tolerant mice

### Table I

| Spleen cells injected neonatally (Mls) | Cells tested for tolerance | Positive cells* |
|----------------------------------------|---------------------------|-----------------|
|                                        | Age | Tissue       | Mice | KJ16 (Vp8) | 44-22-1 (Vp6) |
|                                        |     |              |      |            |               |
| None (-)                               | 8 wk| Lymph node  | 2    | 18.6       | 9.7           |
| DBA/2 (a)                              | 8 wk| Lymph node  | 1    | 14.4       | 0.3           |
| DBA/2 (a)                              | 8 wk| Lymph node  | 1    | 16.8       | 0.7           |
| DBA/2 (a)                              | 8 wk| Lymph node  | 1    | 17.5       | 3.4           |
| None (-)                               | 11 wk| Lymph node | 1   | 20.7       | 10.9          |
| DBA/2 (a)                              | 11 wk| Lymph node| 1    | 16.1       | 0.3           |
| DBA/2 (a)                              | 11 wk| Lymph node| 1    | 16.5       | 0.6           |
| DBA/2 (a)                              | 11 wk| Lymph node| 1    | 14.2       | 0.0           |
| BALB/c (b)                             | 15 d| CRT          | 5    | 23.4 (25.7)\textsuperscript{f} | 14.2 (10.6)\textsuperscript{f} |
| DBA/2 (a)                              | 15 d| CRT          | 6    | 18.1 (20.9)\textsuperscript{f} | 1.5 (0.3)\textsuperscript{f} |
| BALB/c (b)                             | 7 d | CRT          | 1    | 21.5       | 12.4          |
| DBA/2 (a)                              | 7 d | CRT          | 5    | 17.3       | 0.8           |
| BALB/c (b)                             | 15 d| CRT          | 5    | 22.3       | 13.0          |
| DBA/2 (a)                              | 15 d| CRT          | 5    | 17.2       | 0.8           |

* Calculated after subtraction of background staining with fluorescent conjugate alone (compare Fig. 1).

\textsuperscript{f} Data in parentheses obtained after 5 d in culture with PMA/Ionomycin/IL-2.
occurred intrathymically or post-thymically, we therefore treated young (1-2 wk old) neonatally tolerant mice with hydrocortisone and analyzed the resulting CRT for expression of V₆ and V₈. The data from three such experiments involving a total of 16 tolerant mice are summarized in Table I. It is clear that CRT from neonatally tolerant (DBA/2-injected) BALB/c mice had drastically reduced levels of V₆⁺ cells (0.8-1.5%) as compared with controls injected with syngeneic BALB/c spleen cells (13-14% V₆⁺). As was the case with peripheral T cells, expression of V₈ was only marginally reduced as a result of tolerance induction (17-18% vs. 22-23% in the control). Since CRT represent a thymic subpopulation phenotypically and functionally equivalent to mature (CD4⁺ or CD8⁺) T cells (10), these data suggest that the elimination of V₆⁺ cells in neonatally tolerant mice occurs during the normal process of intrathymic repertoire selection.

Several potential artifacts were excluded as an explanation for the apparent disappearance of V₆⁺ T cells after neonatal tolerance induction. First, the unlikely possibility that TCR using V₆ were modulated (rather than eliminated) after contact with antigen in vivo was addressed by culturing the tolerant CRT for 5 further days in the presence of nonspecific stimuli. The resulting cells expressed the same TCR phenotype (0.3% V₆⁺ vs. 20.9% V₈⁺) as the starting population (Table I). Second, the possibility that extensive chimerism resulted in a dilution of V₆⁺ (BALB/c) T cells by V₆⁻ (DBA/2) T cells (4) was ruled out by phenotypic analysis (Table II). Since BALB/c and DBA/2 mice differ at the Lyt-1 locus, it could be concluded that CRT from neonatally tolerant BALB/c mice contained <1% donor-derived DBA/2 T cells.

The clear-cut evidence presented here for a clonal deletion mechanism of neonatal tolerance to Mls⁺ antigens confirms and extends earlier limiting dilution data indicating a dramatic reduction in functional Mls⁺-specific T cell precursors in tolerant mice (9). However the mechanism(s) responsible for elimination of self-reactive clones remains obscure. In this regard, significant chimerism of bone marrow-derived nonlymphoid cells has been detected in the thymus of neonatally tolerant mice (11). Such chimerism may be important in the development and/or maintenance of the tolerant state. Alternatively, it is possible that some processed product of the Mls⁺ gene may recirculate to the neonatal thymus (12) and participate in the in-

### Table II

| Strain (Lyt phenotype) | Spleen cells injected neonatally (Mls) | Positive CRT* |
|------------------------|---------------------------------------|---------------|
|                        |                                       | Exp. 1        | Exp. 2 |
|                        |                                       | Lyt-1 | Lyt-1.1 | Lyt-1 | Lyt-1.1 |
| DBA/2 (Lyt-1.1)        | None (-)                              | 97.2  | 94.5    | ND    | 93.5   |
| BALB/c (Lyt-1.2)       | BALB/c (b)                            | 97.0  | 0.6     | ND    | 0.1    |
| BALB/c                 | DBA/2 (a)                             | 96.9  | 0.5     | ND    | 0.4    |

* After subtraction of background staining with fluorescent anti-rat Ig (Lyt-1) or anti-mouse Ig (Lyt-1.1) conjugate.
duction of tolerance by associating with appropriate host components, in analogy with models of presentation of immunogenic peptides by MHC class II molecules (13). Whatever the explanation, the model system described here should facilitate analysis of the cellular and molecular events associated with the establishment of immunological tolerance.

Summary

The cellular basis of neonatally induced T cell tolerance has been investigated in a model system in which usage of a particular TCR V_\beta segment (V_{\beta 6}) is strongly correlated with reactivity to antigens encoded by the Mls'^ genetic locus. Expression of V_{\beta 6} by peripheral T cells was virtually abolished in BALB/c (H-2^d, Mls'^) mice rendered neonatally tolerant to DBA/2 (H-2^b, Mls'') lymphoid cells, whereas control V_{\beta 8}-bearing T cells remained at near normal levels. Further analysis revealed that elimination of V_{\beta 6}^+ T cells occurred in the thymus of neonatally tolerant mice and could not be explained by receptor modulation or T cell chimerism. These data thus support the clonal deletion model of tolerance induction.

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