CLONAL DELETION OF SELF-REACTIVE T CELLS IN IRRADIATION BONE MARROW CHIMERAS AND NEONATALLY TOLERANT MICE
Evidence for Intercellular Transfer of Mls

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The T cell repertoire is shaped by products of the MHC and by other self antigens. Presumed positive selection favors TCRs that are restricted to self MHC products (1–4); during a subsequent negative selection process T cells reactive to self antigens such as Mls (recently also designated Mls-1) are eliminated before they enter the thymic medulla (5, 6). Several experimental models suggest that induction of tolerance is MHC restricted (7, 8). In contrast, analyses of T cell reactivity against Mls suggest that Mls recognition is less closely correlated with class II MHC allele-specific restriction, although it clearly depends upon the presence of I-E products (9, 10). Because Mls is recognized together with I-E molecules of different MHC haplotypes and since the reacting T cells expressing Vα6 (5) or Vα8.1 (6) are present in most Mls-negative inbred mouse strains studied, it is expected that tolerance to Mls is not MHC restricted.

In this report, we used the mAb 44-22-1 that is specific for Vα6 (11) and detects a T cell subset reactive to Mls (5) to analyze T cell recognition and mechanisms of induction and maintenance of tolerance in chimeras and neonatally tolerant mice exhibiting Mls and the permissive I-E on distinct cell populations. The data suggest that Mls may be transferred between cell compartments and that induction of tolerance to Mls requires presence of I-E but is I-E allele independent.

Materials and Methods

Animals. Inbred BALB/c (H-2k), B10.D2 (H-2d), DBA/2 (H-2b), and CBA/J (H-2k) mice were purchased from the Institute für Zuchthygiene, University of Zürich, Switzerland. DBA/1 (H-2b) and B10.G (H-2b) mice were obtained from Olac, Bicester, Oxon, U.K.

Chimeras. Bone marrow recipients were lethally irradiated (950 rad, 117 rad/min, 137Cs source) and reconstituted 1 d later with 5–20 × 10⁶ T cell-depleted bone marrow or fetal liver cells (1). The transplanted mice had a survival rate of 80–95%. Chimerism was monitored by FACS analysis with anti-H-2 class I mAbs.

Cyttofluorographic Analysis. Aliquots of 10⁶ lymphnode cells were stained at 4°C with rat...
mAbs 44-22-1 (anti-V\textsubscript{66}) (11) or KJ16-133 (anti-V\textsubscript{88.1}/V\textsubscript{88.2}) (6) followed by fluorescent goat anti-rat second reagent. Cortisone-resistant thymocytes (CRT; obtained 48 h after injection of 4 mg i.p. of hydrocortisone) were found to express comparable percentages of V\textsubscript{66} and V\textsubscript{88}. For H-2 typing, nylon wool-purified spleen cells were incubated at 20°C with mAbs 100-5.28 (anti-K\textsuperscript{D\textsuperscript{d}}) and 34-1-2 (anti-K\textsuperscript{D\textsuperscript{d}}) (12). Samples were analyzed on an EPICS Profile flow cytometer (Coulter Electronics, Inc., Hialeah, FL).

\textsuperscript{3}Cr-release Assay. Vaccinia virus (Lancy strain; Schweizerisches Serum und Impfinstitut, Bern, Switzerland) was injected intravenously in a dose of 3 x 10\textsuperscript{6} PFU. Infected mice were killed 6 d later and single spleen cell suspensions were tested for CTL activity on virus-infected and uninfected \textsuperscript{3}Cr-labeled target cells as described in detail elsewhere (1, 13).

Results and Discussion

Characterization and Examination of V\textsubscript{66} Usage in Bone Marrow Chimeras. In BALB/c → DBA/2 allogeneic chimeras where Mls\textsuperscript{b} (H-2\textsuperscript{d}) stem cells were used to reconstitute Mls\textsuperscript{a} (H-2\textsuperscript{b}) mice only low levels of V\textsubscript{66}+ T cells were found (Table I). Therefore host-derived radioresistant cells were capable of inducing tolerance to Mls\textsuperscript{a}. The converse combination, i.e., DBA/2 (Mls\textsuperscript{a}) → BALB/c (Mls\textsuperscript{b}), showed that Mls\textsuperscript{a} expression by lymphohemopoietic cells alone was also sufficient to induce tolerance. The same conclusions may be derived from observations in F\textsubscript{1}→parent chimeras (Table II). Thus, irradiated mice retain their ability to express Mls\textsuperscript{a} and tolerogenic Mls\textsuperscript{a} can be provided by chimeric donor- and host-type cells.

The lower percentages of V\textsubscript{66}+ or V\textsubscript{88}+ cells in bone marrow chimeras compared

### Table I

| Bone marrow donor | Bone marrow recipient | Donor | Recipient | Percent lymph node cells expressing |
|-------------------|-----------------------|-------|-----------|------------------------------------|
| BALB/c            | DBA/2 (4)             | d b   | d a       | V\textsubscript{66} (Ab 44-22-1)   |
|                   |                       |       |           | 0.8 ± 0.4                           |
|                   |                       |       |           | 10.4 ± 0.4                           |
|                   | DBA/2 (4)             | d b   | d a       | V\textsubscript{88} (Ab KJ16)       |
|                   |                       |       |           | 7.4 ± 0.3                            |
|                   | DBA/c (4)             | d b   | d b       | V\textsubscript{66} (Ab 44-22-1)   |
|                   |                       |       |           | 4.3 ± 0.5                           |
|                   |                       |       |           | 7.7 ± 0.5                            |
|                   | DBA/2 (4)             | d a   | d a       | V\textsubscript{88} (Ab KJ16)       |
|                   |                       |       |           | 0.8 ± 0.4                           |
|                   |                       |       |           | 8.1 ± 0.9                            |
| B10.G (2)         | B10.G (2)             | q b   | q b       | V\textsubscript{66} (Ab 44-22-1)   |
|                   |                       |       |           | 2.4 ± 0.1                           |
|                   |                       |       |           | 5.3 ± 0.6                            |
| DBA/1 (2)         | DBA/1 (2)             | q a   | q a       | V\textsubscript{88} (Ab KJ16)       |
|                   |                       |       |           | 2.5 ± 0.1                           |
|                   |                       |       |           | 5.0 ± 0.0                            |

Chimeras were prepared as described in Materials and Methods and killed between 6 and 12 wk after transplantation. The numbers of transplanted mice analyzed are indicated in parentheses. Lymph nodes were used to prepare samples for FACS-analyses. The mean percentages and SEM of positive cells are given following subtraction of background values staining with the fluorescent anti-Ig conjugate alone. Total lymph node cell preparations contained between 50 and 65% Thy-1\textsuperscript{+} cells. Expression of Lyt-1.1 was analyzed to trace DBA/2-derived cells: BALB/c → DBA/2 chimeras had 0.6% Lyt-1.1\textsuperscript{+} lymph node cells (Lyt-1.1\textsuperscript{+}: 93.3 ± 1.5%). DBA/2 → BALB/c chimeras had 91.3 ± 1.2% Lyt-1.1\textsuperscript{+} lymph node cells (Lyt-1.1\textsuperscript{+}: 87.3 ± 2.9%). H-2 and Mls typing as well as expression of I-A/I-E molecules were taken from the literature (17, 18).
with untreated mice was partially because of the decreased proportion of T cells in lymph node preparations (~50 vs. 65% Thy-1+ cells); analysis was performed relatively soon after reconstitution of the chimeras. Since the lower percentages were found in syngeneically and semiallogeneically reconstituted animals, it probably reflects radiation damage and/or less efficient T cell maturation in chimeras.

**Evidence for Intercellular Transfer of Mlsa in Irradiation Bone Marrow Chimeras.** H-2q mice possess V$\gamma$6+ T cells, irrespective of Mlsa expression (Table I); the same is found in syngeneic control chimeras of this haplotype. Using H-2d/I-E+ and H-2q/I-E- mice we prepared F1-parent chimeras in which Mlsa was only expressed by H-2q-bearing (DBA/1) cells (Table II). Such cells lack I-E molecules and do not efficiently present Mlsa for either stimulatory response or for tolerance induction (9). These chimeras eliminated V$\gamma$6+ T cells, demonstrating that for induction of tolerance Mlsa and I-E antigens may be provided by distinct cell subsets. Therefore, Mlsa had to be transferred to appropriate APCs.

**I-E- Mlsa Spleen Cells Induce Neonatal Tolerance in I-E+ Mlsa Recipients.** The capacity of Mlsa spleen cells to induce neonatal tolerance was tested on (BALB/c × B10.G)F1 newborn mice; they were injected within 24 h of birth with 100 × 10e6 spleen cells of DBA/1 or DBA/2 mice. 2 wk later we analyzed V$\gamma$6 and V$\beta$8 expression on CRT (Table III). As expected, neonatal injection of I-E/-/Mlsa spleen cells (DBA/2) severely reduced V$\gamma$6 expression in Mlsa recipients. Interestingly, I-E-/Mlsa spleen cells (DBA/1) reduced the expression of V$\gamma$6 almost equally well, indicating that neonatal tolerance was induced to a considerable degree by transfer of Mlsa from I-E- donor spleen cells to I-E+ APCs of the recipient.

The presented data confirm recent experiments by Pullen et al. (14), using a different mAb specific for V$\beta$3 that correlates with reactivity to Mlsa. Analysis of A (Mlsa, class II nonpermissive) + B (Mlsa, class II permissive)→AxB (Mlsa) irradiation bone marrow chimeras revealed absence of Mlsa-specific V$\beta$3+ T cells in these chimeras. Our results are also compatible with in vitro studies published by DeKruyff et al. (15) showing that Mlsa-specific T cell clones proliferated when cocultured with DBA/1 stimulator B cells only if I-E+ splenic adherent cells were added.
Spleen cells from chimeras or control mice:

| Bone marrow donor | Bone marrow recipient | Killer/target ratio | Percent specific $^{51}$Cr release of vaccinia virus-infected target cells |
|-------------------|-----------------------|---------------------|--------------------------------------|
| (DBA/2 × B10.G)F1 | DBA/2                 | 30                  | H-2$^d$(D2) 3  |
|                   |                       | 10                  | H-2$^q$(DBA/1) 1  |
|                   |                       | 3                   | 2  |
| (DBA/1 × DBA/2)F1 | B10.G                 | 30                  | H-2$^q$(D2) 92 |
|                   |                       | 10                  | 45 |
|                   |                       | 3                   | 7  |
| (DBA/1 × DBA/2)F1 | B10.D2                | 30                  | H-2$^q$(D2) 7 |
|                   |                       | 10                  | 0  |
|                   |                       | 3                   | 2  |
| (BALB/c × B10.G)F1| DBA/2                 | 30                  | H-2$^d$(D2) 7 |
|                   |                       | 10                  | 0  |
|                   |                       | 3                   | 2  |
| DBA/2             |                       | 30                  | H-2$^d$(D2) 4 |
|                   |                       | 10                  | 4  |
|                   |                       | 3                   | 1  |
| B10.G             |                       | 30                  | H-2$^q$(D2) 49 |
|                   |                       | 10                  | 4  |
|                   |                       | 3                   | 1  |

Test duration was 5 h; spontaneous release from infected D2, 15%; DBA/1, 19%.

Dominant Restriction Specificity of T Cells Does Not Influence Mls$^a$-dependent TCR V$\beta$6 Usage in Chimeras. In the presence of Mls$^a$, H-2$^d$ mice delete V$\beta$6$^+$ T cells, whereas in H-2$^q$ mice, ~3–4% of mature T cells express V$\beta$6 (Table I). To evaluate the influence of the restriction specificity of T cells on tolerance induction, irradiation bone marrow chimeras of the following general type were made: (H-2$^d$ × H-2$^q$)-F1/Mls$^{ab}$ or Mls$^{aa}$ or Mls$^{aax}$ stem cells were used to reconstitute H-2$^d$/Mls$^a$ or H-2$^q$/Mls$^a$ or H-2$^d$/Mls$^b$ or H-2$^q$/Mls$^b$ irradiated recipients. The mice were typed for TCR V$\beta$6 expression (Table II) and for effector T cell restriction specificity (Table IV). After infection with vaccinia virus the bone marrow chimeras expressed anti-
viral T cell immunocompetence restricted predominantly to the H-2 haplotype of the thymus. In presence of Mls<sup>a</sup> and I-E the chimeras had reduced levels of V<sub>ß</sub><sup>6</sup><sup>+</sup> cells (Table II), no matter whether their T cells were restricted to H-2<sup>d</sup> or H-2<sup>d'</sup>. Therefore, selection of V<sub>ß</sub><sup>6</sup><sup>+</sup> T cells apparently did not depend on the restriction specificity of effector T cells determined by the thymus. We obtained similar results with chimeras developing effector T cells restricted to H-2<sup>d</sup> (data not shown). Thus, tolerance induction to Mls<sup>a</sup> is not I-E allele restricted, but generally I-E dependent.

The rules of Mls<sup>a</sup> recognition and induction of tolerance shown here are compatible with the earlier findings that in H-2<sup>d</sup> and H-2<sup>d'</sup> mice I-A is not involved, and the presence of I-E is necessary and sufficient for Mls<sup>a</sup> recognition independent of the I-E allele (9,16). The fact that Mls<sup>a</sup> obviously does not exhibit the typical characteristics of T cell antigens, but still requires MHC molecules for presentation, may be called “pseudo-restriction” in contrast to the usually allele-specific restricted T cell recognition. This may be due to the low degree of polymorphism of I-E molecules (17).

In conclusion, our experiments are in agreement with the hypothesis of Mls<sup>a</sup> being a peptide: as a whole or as fragments thereof it may be shed, reprocessed, and/or bound to I-E antigens. What remains unclear is why and how Mls<sup>a</sup> peptides or Mls<sup>a</sup> fragments stimulate such a high proportion of T cells.

**Summary**

Tolerance to Mls<sup>a</sup> has been shown to be associated with clonal deletion of cells carrying TCR ß chain variable regions V<sub>ß</sub>6 or V<sub>ß</sub>8.1 in mice possessing I-E antigens. To evaluate the rules of tolerance induction to Mls<sup>a</sup> we prepared irradiation bone marrow chimeras expressing Mls<sup>a</sup> or Mls<sup>b</sup> and I-E by different cell types. Deletion of V<sub>ß</sub>6<sup>+</sup>, Mls<sup>a</sup>-reactive T cells required the presence of Mls<sup>a</sup> and I-E products either on bone marrow-derived cells or on irradiated recipient cells. Tolerance was induced when Mls<sup>a</sup> and I-E were expressed by distinct cells of the chimera. Also neonatally tolerized mice exhibited depletion of V<sub>ß</sub>6<sup>+</sup> cells after injection of I-E<sup>+</sup> Mls<sup>a</sup> spleen cells (DBA/1) into newborn I-E<sup>+</sup> Mls<sup>b</sup> mice (BALB/c × B10.G)F<sub>1</sub>. These results suggest that the product of the Mls<sup>a</sup> locus is soluble and/or may be transferred from cell to cell and bound to I-E antigens.

The chimera experiments also showed that tolerance to Mls<sup>a</sup> is H-2 allele independent, i.e., is apparently unrestricted. Differentiation of chimeric (H-2<sup>d</sup>/Mls<sup>a</sup> × H-2<sup>d'</sup>/Mls<sup>b</sup>)F<sub>1</sub> stem cells in either an H-2<sup>d</sup> or an H-2<sup>d'</sup> thymus revealed that tolerance assessed by absence of V<sub>ß</sub>6<sup>+</sup> T cells is not dependent on the thymically determined restriction specificity of T cells.

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