Peripheral T Cell Activation and Deletion Induced by Transfer of Lymphocyte Subsets Expressing Endogenous or Exogenous Mouse Mammary Tumor Virus

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Summary
Murine T cell reactivity with products of the minor lymphocyte stimulatory (Mls) locus correlates with the expression of particular variable (V) domains of the T cell receptor (TCR) β chain. It was recently demonstrated that Mls antigens are encoded by an open reading frame (ORF) in the 3' long terminal repeat of either endogenous or exogenous mouse mammary tumor virus (MMTV). Immature thymocytes expressing reactive TCR-Vβ domains are clonally deleted upon exposure to endogenous Mtv's. Mature T cells proliferate vigorously in response to Mls-1a (Mtv-7) in vivo, but induction of specific anergy and deletion after exposure to Mtv-7-expressing cells in the periphery has also been described. We show here that B cells and CD8+ (but not CD4+) T cells from Mtv-7+ mice efficiently induce peripheral deletion of reactive T cells upon transfer to Mtv-7- recipients, whereas only B cells stimulate specific T cell proliferation in vivo. In contrast to endogenous Mtv-7, transfer of B, CD4+, or CD8+ lymphocyte subsets from mice maternally infected with MMTV(SW), an infectious homologue of Mtv-7, results in specific T cell deletion in the absence of a detectable proliferative response. Finally, we show by secondary transfers of infected cells that exogenous MMTV(SW) is transmitted multidirectionally between lymphocyte subsets and ultimately to the mammary gland. Collectively our data demonstrate heterogeneity in the expression and/or presentation of endogenous and exogenous MMTV ORF by lymphocyte subsets and emphasize the low threshold required for induction of peripheral T cell deletion by these gene products.

T cell reactivity to antigens of the Mls locus correlates with expression of certain TCR-Vβ domains (1-5). The Mls loci have recently been shown to be encoded by open reading frames (ORF)1 in the 3' long terminal repeat of different mouse mammary tumor viruses (MMTV) (6-8). Expression of Mtv-7, an endogenous proviral MMTV locus that determines the Mls-1a phenotype (9), leads to the intrathymic deletion of cells expressing TCR-Vβ6, 7, 8,1, or 9 (1-5). Recently, we characterized an infectious equivalent of Mtv-7, called MMTV(SW) (10), that exhibits the same TCR-Vβ specificity. MMTV(SW) and Mtv-7 ORF molecules are almost identical in their carboxy termini, a polymorphic region that has been implicated in determining TCR-Vβ specificity (11). The availability of MMTV(SW) thus provides a unique opportunity to compare T cell responses to endogenous and exogenous MMTV ORF products.

In contrast to immature thymocytes, mature T cells respond by proliferation upon exposure to MMTV ORF products. In the case of Mtv-7, this initial response is followed paradoxically by functional inactivation (anergy) and even, in some cases, by clonal deletion of the reactive T cells (12-17). Very little is known about the mechanisms leading to peripheral anergy and deletion of Mtv-7-reactive T cells, and it is still unclear whether these are causally related phenomena. Even less is known about exogenous MMTV, although there is evidence that lymphocytes may be involved in its transmission to the mammary gland (18).

In an attempt to clarify some of these issues, we have injected highly purified lymphocyte subsets (B, CD8+, or CD4+ cells) from mice carrying the endogenous Mtv-7 or the exogenous MMTV(SW), into normal recipients and monitored their ability to elicit clonal expansion or deletion of

1 Abbreviations used in this paper: MMTV, mouse mammary tumor virus; ORF, open reading frame.
the reactive T cells. Furthermore, we have used secondary transfer experiments to investigate the ability of these subsets to infect each other and ultimately lead to maternal transmission of the virus.

Materials and Methods

Mice. BALB/c, CBA/J, and CBA/Ca mice were obtained from Harlan Olac Ltd. (Bicester, UK) and BALB.D2.Mls-1 mice were maintained from breeding pairs originally provided by Dr. H. Fetschenstein (London Hospital Medical College, London, UK) (19). Mice that were maternally infected with MMTV (SW) (BALB/c [SW]) were originally obtained from IFFA Credo (L'Arbresle, France) and subsequently maintained in our colony. For thymectomy, mice (5–6 wk of age) were anesthetized by intraperitoneal injection of 600 μl of 0.04 M 2.2.2-tribromoethanol (1 g/ml in isooamyl alcohol subsequently diluted 80× in PBS; Merck, Darmstadt, Germany). Thymectomy was performed by suction via a transcervical-rosternal incision. Experiments were begun after the mice had rested for 7 d.

Antibodies. The following antibodies were used in this study: anti- Vα6 (44.22.1) (20), anti-V8.2 (F23.2) (2), anti-Vγ14 (14.2) (21), anti-CD4 (OKT4) (22), anti-CD8 (53-6.7) (23), and anti-Thy-1 (AT83) (24).

Cell Preparations. Suspensions of CD4+ and CD8+ T cells were prepared from lymph node, and B cells and CD4+ or CD8+ T cells were from spleen. CD4+ or CD8+ T cells were first positively selected (to >95% purity) using directly coupled anti-CD4 or anti-CD8 microbeads and a magnetic cell separation device (MACS®; Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) (25). These cells were stained with fluorescently labeled (either FITC or PE) anti-CD4 or anti-CD8. B cells were enriched from spleen by anti-Thy-1 plus complement treatment and stained with a polyclonal rabbit F(ab)2 anti-mouse Ig FITC conjugate. These preparations were further purified to >99.5% homogeneity by sorting using a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA). Peripheral blood was taken from the tail vein and mononuclear cells were isolated by centrifugation of heparinized samples (diluted with PBS-FCS) on a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) cushion.

Flow Cytometry. Lymph node cells or PBMC were labeled in a single step with direct FITC conjugates of either anti-Vα6, anti-Vγ8.2, or anti-Vγ14, together with anti-CD4-PE (Becton Dickinson & Co.). Monitoring of the levels of Vα6+ cells among CD4+ T cells in blood samples was controlled in each case by measurement of Vα14+ cells in the same preparation. All samples were analyzed using a FACScan® and the Lysys II program (Becton Dickinson & Co.). Dead cells were excluded by a combination of forward and side scatter.

Cell Transfers. Mice were injected in one hind footpad with the indicated numbers of purified cells in 25 μl of PBS. Control mice were either not injected or received 25 μl of PBS or syngeneic cells.

Results

Local Vα6+ CD4+ T Cell Response Induced by Transfer of Lymphocyte Subsets from Mice Expressing Endogenous Mtv-7 (Mls-1+) or Infectious MMTV(SW). After injection of B cells from Mls-1+ congenic BALB/c mice (BALB.D2; Mtv-7+, H-2d) into the hind footpad of BALB/c (Mtv-7−) mice, we observed a strong and specific increase in the number of TCR-Vα6+ CD4+ cells in the popliteal lymph node, which peaked between 3 and 5 d after injection. Given the dominance of TCR-Vα6+ CD4+ cells relative to other Mls-1+-reactive TCR-Vα6+ in responses to Mls-1+ (26), we confined study of reactive cells to this population. The specificity of the response was ensured by following the frequencies of cells expressing nonreactive TCR-Vα6 domains (either Vα8.2 or Vγ14). Around 25% of total CD4+ T cells expressed Vα6 compared with ~11% in control mice (Table 1). This response was only induced by B cells and not by purified (>99.5% pure) CD4+ or CD8+ T cells from Mtv-7− mice. Since a previous report (15) showed that CD8+ T cells were also able to induce proliferation in mice of the H-2k haplotype (after intravenous injection into thymectomized recipients), we injected either purified B cells or CD8+ T cells from CBA/J (Mtv-7+, H-2k) into the footpads of CBA/Ca (Mtv-7−, H-2k) mice. Again, B cells efficiently stimulated a Vα6+ CD4+ T cell response whereas CD8+ T cells did not (Table 1).

Similarly, we analyzed the response of BALB/c mice to purified B cells, CD8+ T cells, and CD4+ T cells from >6-mo-old BALB/c mice neonatally infected with MMTV(SW) (hereafter referred to as BALB/c [SW]). Infection of donor mice was verified by the absence of TCR-Vα6+ cells in the periphery before isolation of lymphocyte populations. In each case no proliferative response was detected (Table 1).

Deletion of Vα6+ CD4+ T Cells Induced by Transfer of Lymphocyte Subsets from Mice Expressing Endogenous Mtv-7 or Infectious MMTV(SW). In experiments conducted in parallel with the above stimulation studies, we monitored TCR-Vα6+ CD4+ cells in the peripheral blood in recipient mice injected with the purified cell subsets. In all experiments, the specificity of deletion was verified by monitoring the levels of Vα14+ CD4+ T cells. This control population remained largely unaffected in all experiments, except for slight, compensatory increases in their percentage due to the deletion of Mtv-7-reactive T cells (data not shown). In agreement with a previous study (27), specific deletion of Vα6+ CD4+ T cells in BALB/c mice was detected as early as 2 wk after injection of either B cells or CD8− T cells but not CD4+ T cells from BALB.D2 Mtv-7+ mice (Fig. 1A). Dose-response and kinetic studies demonstrated that the deletion of TCR-Vα6+ CD4+ cells induced by injection of CD8− T cells was slightly faster and of greater magnitude than that induced by the same number of B cells (Fig. 1, B and C). In all cases we observed continuous deletion over the entire time course (>160 d), suggesting persistence of Mtv-7+ cells in the recipients.

Since the mouse strains used for these experiments were Mls-1+ congenic, it seemed likely that permanent chimerism was established in the relevant subsets. This hypothesis was tested in experiments where B cells, CD8− T cells, and CD4+ T cells were purified from mice that had previously received B cells or CD8− T cells from BALB.D2 mice, and transferred into new host BALB/c mice. In these secondary transfers, deletion was only induced by the CD8− T cell subset and not by any other subset from mice that had received CD8+ T cells in the primary transfer (Fig. 2A). However, no subset (including B cells) from mice that had received BALB.D2 B cells originally was able to induce dele-
Table 1. In Vivo Response to Cells from Mice Expressing Endogenous Mtv-7 (Mls-1b) or from Mice Maternally Infected with MMTV(SW)

| Responder strain | Donor strain | Injected subset | CD4+ T cell subset* |
|------------------|--------------|-----------------|--------------------|
|                  |              |                 | Vβ6 | Vβ8.2 | Vβ14 |
| BALB/c (Mls-1b)  | BALB.D2 (Mls-1b) | B                | 23.5 ± 3.8 | 11.6, 11.1 | 8.1 ± 0.2 |
|                  |              | CD6             | 11.6 ± 1.1 | 13.2 ± 0.3 | 9.7 ± 0.2 |
|                  |              | CD4             | 13.5 ± 2.7 | 12.6, 12.7 | 9.1 ± 0.4 |
| BALB/c (SW)      | B            |                 | 12.1, 11.9 | 13.4, 13.4 | 9.5, 9.9 |
|                  | CD8          |                 | 11.3, 12.0 | 13.6, 14.8 | 9.4, 9.1 |
|                  | CD4          |                 | 12.1, 13.0 | 13.7, 14.0 | 9.4, 9.7 |
| BALB/c (Mls-1b)  | B            |                 | 11.9, 11.8 | 13.3, 12.8 | 10.0, 10.0 |
|                  | CD8          |                 | 11.3, 11.4 | 12.8, 14.0 | 9.8, 9.6 |
|                  | CD4          |                 | 11.9, 12.1 | 13.0, 13.0 | 9.9, 9.8 |
| No injection     |              |                 | 11.5 ± 0.6 | 13.4 ± 0.4 | 9.8 ± 0.3 |
| CBA/CaH (Mls-1b) | CBA/J (Mls-1b) | B                | 21.6, 27.4 | ND     | 5.2, 5.0 |
|                  |              | CD8             | 12.2, 12.7 | ND     | 6.5, 6.5 |
| No injection     |              |                 | 11.0 ± 0.5 | ND     | 6.8 ± 0.2 |

* Draining lymph node cells were taken 3–4 d after injection of 10⁶ cells of the indicated subsets and assessed for the expression of the different TCR-Vβ domains by flow cytometry. Results from individual mice are presented, or are given as the mean ± SD where three or more mice were independently examined. Significantly increased percentages are shown in boldface.

Deletion of Reactive T Cells Occurs Both Intrathymically and in the Periphery. The deletion of peripheral T cells detected in the blood may have been a measure of ongoing intrathymic negative selection and thus dependent on homing of the injected subsets to the thymus. Consistent with this, deletion of TCR-Vβ6+CD4+ cells was observed in the thymuses of BALB/c mice injected with BALB.D2 B cells or CD8+ T cells (data not shown). However, transfer of B cells or CD8+ T cells from either BALB.D2 or BALB/c(SW) mice into thymectomized BALB/c recipients induced deletion of TCR-Vβ6+CD4+ cells, demonstrating extrathymic peripheral T cell deletion (Fig. 5).

Transfer of MMTV(SW)-infected Lymphocyte Subsets Leads to Mammary Gland Infection. Since MMTV is maternally transmitted, recipient BALB/c mice, in which deletion of TCR-Vβ6+CD4+ cells was observed after second-round transfer of infected lymphocyte subsets (described above), were bred with normal BALB/c males. Offspring were bled at 4–5 wk of age and the levels of TCR-Vβ6+CD4+ cells were followed to determine if milk transmission of MMTV(SW), and thus mammary gland infection, had occurred. In all the tested subset combinations of primary and secondary transfers, we observed deletion of TCR-Vβ6+CD4+ cells in pups demonstrating efficient infection of the mammary gland (Table 2).

Discussion

Previous work has suggested that superantigen-triggered deletion of mature T cells from the peripheral pool is associated with a prior expansion phase (15, 16, 29, 30). However, we
Figure 1. Kinetics of deletion of V\(\beta\)6\(^+\)CD4\(^+\) T cells of BALB/c mice injected with BALB.D2.Mls-1\(^a\) cells. Blood samples were taken at the indicated time points after footpad injection. (A) Injection of 10\(^6\) cells of each subset: B cells (○), CD8\(^+\) T cells (■), CD4\(^+\) T cells (□), or no injection (△). Each curve represents data from at least three mice (mean ± SD). (B) Deletion dose response after injection of BALB.D2.Mls-1\(^a\) B cells: 10\(^6\) cells (■), 3 \times 10\(^5\) cells (□), 10\(^5\) cells (○), 3 \times 10\(^4\) cells (△), no injection (△). Each curve represents data from two mice. (C) Deletion dose response after injection of BALB.D2.Mls-1\(^a\) CD8\(^+\) T cells: 10\(^6\) cells (■), 3 \times 10\(^5\) cells (□), 10\(^5\) cells (○), 3 \times 10\(^4\) cells (△), 10\(^4\) cells (△), or no injection (△). Each curve represents data from two mice.

Figure 2. Kinetics of deletion of V\(\beta\)6\(^+\)CD4\(^+\) T cells induced by secondary transfer of lymphocyte subsets from some primary recipients of BALB.D2.Mls-1\(^a\) cells. B cells (○), CD8\(^+\) T cells (■), and CD4\(^+\) T cells (□) were purified from mice in which V\(\beta\)6\(^+\)CD4\(^+\) cells had been deleted after receiving either BALB.D2.Mls-1\(^a\) CD8\(^+\) T cells (A) or B cells (B). 10\(^6\) cells of each subset was transferred into secondary BALB/c recipients at >110 d after primary transfer. The levels of V\(\beta\)6\(^+\)CD4\(^+\) T cells in peripheral blood of the secondary recipients were monitored at the indicated time points. Each curve represents data from at least three mice (mean ± SD).

Our data indicate significant variation with respect to the ability of different cell types to present Mls-1\(^a\) in a form that is able to induce proliferation and/or deletion of the reactive T cell responses to endogenous and exogenous viral superantigens.
V\textsubscript{86} CD4\textsuperscript{+} T cells. An essential requirement for Mls-1\textsuperscript{a} presentation would, logically, be the expression of Mtv-7 ORF transcripts, which were detectable in all subsets using a PCR-based strategy (28, and our unpublished data). Clearly, constitutive expression of class II MHC is also required for the induction of proliferation of the ORF-reactive T cells since B cells but neither CD4\textsuperscript{+} nor CD8\textsuperscript{+} T cells (both generally accepted to be class II MHC negative in the mouse), were able to stimulate a classical Mls-1\textsuperscript{a} response after transfer. However, the factors determining the capacity of a given cell type to present Mls-1\textsuperscript{a} for the induction of peripheral clonal deletion are much less clear. Consistent with a previous report (27), both B cells and CD8\textsuperscript{+} T cells induced deletion, while similar doses of CD4\textsuperscript{+} T cells did not.

In considering this unexpected dichotomy between CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, it is conceivable that an MHC class II-negative cell expressing Mtv-7 can induce deletion by first transferring ORF products to an MHC class II-positive cell. Such transfers of molecules from CD8\textsuperscript{+} T cells have in fact been reported in other systems, i.e., for the lysosomal enzymes α-d-mannosidase and β-glucuronidase (34, 35). In the case of ORF products this may require transfer of only small amounts of the antigen since deletion is a more sensitive readout than proliferation (31-33), which may explain the failure to detect transfer previously in one (36) but not in other reports (37, 38). Alternatively, it is possible that the injected CD8\textsuperscript{+} T cells passively acquire class II MHC molecules (39) more efficiently than CD4\textsuperscript{+} cells, enabling them to directly present ORF product to reactive T cells. A final consideration for the putative transfer of ORF products is that it would probably require continuous exposure to ORF molecules, since B cells from a mouse that received CD8\textsuperscript{+} T cells (BALB.D2.Mls-1\textsuperscript{a}) did not stably acquire the Mtv-7\textsuperscript{+} phenotype and induce deletion upon retransfer.

The greater deletion-inducing capacity of CD8\textsuperscript{+} T cells relative to B cells from Mls-1\textsuperscript{a}-expressing mice raises the possibility that CD8\textsuperscript{+} T cells may proliferate or survive better than B cells after transfer to the host animal. This point is supported by the secondary subset transfers in which only CD8\textsuperscript{+} T cells were able to induce deletion of TCR-V\textsubscript{86} CD4\textsuperscript{+} cells. Therefore, the inability of B cells to induce deletion after a second-round transfer may have been
Peripheral deletion of V_{6}^{+}CD_{4}^{+} T cells induced by injection of BALB.D2.Mls-1 cells or cells from BALB/c(SW) mice. Recipients were thymectomized 1 wk before injection of the indicated subsets. Deletion profiles of mice injected with 10^{6} B cells (●) or CD_{8}^{+} T cells (□) from BALB.D2.Mls-1 mice, or B cells (○) or CD_{8}^{+} T cells (□) from BALB/c(SW) mice, or mice that were not injected (△). Each curve represents data from two mice.

Deletion kinetics induced by the F_{1} subsets relative to BALB.D2 subsets indicated that the F_{1} CD_{8}^{+} T cells (but not the F_{1} B cells) were less efficient than their parental counterparts, suggesting that the capacity to induce deletion may nevertheless have been enhanced by responses to minor histocompatibility antigens (our unpublished observations). Interestingly, comparison of the deletion kinetics induced by the F_{1} subsets relative to BALB.D2 subsets indicated that the F_{1} CD_{8}^{+} T cells were able to induce deletion upon secondary transfer, demonstrating that the establishment of chimerism in the CD_{8}^{+} T cell compartment did not require activation of the donor cells by minor histocompatibility antigens (our unpublished observations). Each of the MMTV(SW)-infected subsets was able to act as a source of infectious virus that could be transmitted to the other lymphocyte subsets and ultimately to the mammary gland. This is consistent with a previous report showing T cell-dependent expression of viral gp52 by mammary gland after transfer of lymphoid cells from MMTV(C3H)-infected mice (18). Interestingly, virus transmission between subsets was slightly less efficient from B cell to B cell, judging by the lower frequency of second-round B cell recipients in which deletion occurred. Whether the efficiency of transmission from one subset to another varies due to different virus receptors.

| Secondary BALB/c recipient (mother) | CD4^{+} T cells | Offspring CD4^{+} T cells |
|------------------------------------|-----------------|--------------------------|
|                                    | V_{6}           | V_{14}       | V_{6}   | V_{14}   |
| B                                  | B               | 1.8          | 11.2   | 6.0 ± 1.7 | 9.5 ± 0.5 |
|                                   | CD8              | 1.7          | 10.5   | 4.3 ± 0.9 | 8.9 ± 0.2 |
|                                   | CD4              | 1.8          | 11.1   | ND       | ND        |
| CD8                                | B               | 1.2          | 12.2   | 2.6 ± 0.4 | 9.8 ± 0.5 |
|                                   | CD8              | 1.4          | 10.5   | 6.0 ± 0.9 | 8.6 ± 0.5 |
|                                   | CD4              | 1.0          | 11.2   | 3.7 ± 0.8 | 10.3 ± 0.3 |
| CD4                                | B               | 2.2          | 12.0   | 5.9 ± 1.2 | 8.5 ± 0.8 |
|                                   | CD8              | 1.4          | 11.3   | 6.2 ± 0.8 | 9.3 ± 0.5 |
|                                   | CD4              | 2.1          | 10.7   | 3.3 ± 0.8 | 9.7 ± 0.4 |
| Normal BALB/c (no transfers)      |                 |             |        | 10.2 ± 0.6 | 9.9 ± 0.7 |

* Primary subset donors were BALB/c (SW) mice.
† Donors for the secondary subset transfers were the BALB/c recipients of the primary transfers. These mice were mated with normal (uninfected) BALB/c mice. Offspring from these pairs were bled at 4–6 wk of age. The percentages of V_{6}^{+} and V_{14}^{+} T cells in the CD_{4}^{+} T cell subset in peripheral blood were determined by flow cytometry. Data are given as mean ± SD of four pups from each litter.

Table 2. Mammary Gland Infection (Milk Transmission) of MMTV(SW) Induced by Transfer of Lymphocyte Subsets from Maternally Infected Mice
on different cell types, to the percentage of virus-infected cells in each subset at the time of transfer, or to the characteristics of the interactions between the subsets can not be determined using this approach. With respect to the latter, the lower efficiency of virus transfer from injected B cells to host B cells may reflect infrequent B-B interactions (as compared with T-B interactions) during which direct virus transfer could take place and/or the requirement for an intermediate virus host (e.g., T cells) for infection of B cells of the recipient animal. Additionally, the dependence of virus transfer on cell-to-cell contact is consistent with the observation that viremia is generally not associated with infection by MMTV and that MMTV can not be transferred by plasma (41). Indeed, using the most sensitive readout known for MMTV infection (i.e., T cell deletion) we have similarly failed to see any evidence for transmission of MMTV(SW) after transfer of plasma or cell-free culture supernatants from infected mice (our unpublished observations). These data argue against transfer of free MMTV particles in the cell injections described here.

The kinetics of infection of the different lymphocyte subsets in maternally infected mice were not addressed in this study (all maternally infected animals were used as donors at ≥6 mo of age). However, from the subset transfer experiments we know that detectable transmission of virus from one lymphocyte subset to another can take place within at least 10 wk (earlier time points were not tested), but this may vary depending on the subset in question. The spread of the virus away from the site of infection (i.e., to the mammary gland and other peripheral lymphoid organs) via migration of infected cells must occur rapidly since deletion of peripheral TCR-V6δ+CD4+ cells can be detected as soon as 4 wk after birth or 2 wk after cell transfers. Furthermore, the breeding of second-round recipients indicated that virus transmission to the mammary gland could occur within 14 wk after transfer of the infected subsets, and probably more rapidly since maternally infected females of 8-9 wk of age can transmit virus to their first litter at this time (data not shown). We have recently obtained results demonstrating viral DNA in B cells but not T cells of draining lymph nodes in adult mice 6 d after virus injection, suggesting that these cells are the primary targets for viral infection (28). The events that follow to bring about infection of other subsets are currently being assessed to determine the pathways taken by the virus to achieve mammary gland infection. The mode of infection of the mammary gland of the adult recipients, either directly by the transferred infected subset or via an intermediate cell type, and the requirement for superantigen-induced T cell responses in this transmission require further investigation.

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