Requirement of Dendritic Cells and B Cells in the Clonal Deletion of Mls-reactive T Cells in the Thymus

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Summary

The present study was performed to identify cells responsible for the elimination of T cells reactive with minor lymphocyte-stimulating (Mls) antigens during T cell development. Experiments were carried out in a fetal thymus organ culture (FTOC) system. To examine the tolerance-inducing activity, various populations of cells from adult CBA/J (Mls-1') mice were injected into deoxyguanosine (dGuo)-treated FTOC of C3H/He (Mls-1b) mice with a microinjector, and 2 d later, the thymus lobes were injected with fetal thymus cells from C3H/He mice as T cell precursors. After 14 d of cultivation, cells were harvested and assayed for the expression of the T cell receptor Vß6 element. The absence or marked reduction of T cells expressing Vß6 at high levels (Vß6high) was regarded as indicating the deletion of Mls-1-reactive T cells.

T cell-depleted populations of thymic as well as splenic cells from CBA/J mice were able to induce clonal deletion. Further characterization of the effector cells was carried out by fractionating the spleen cells before injecting them into dGuo-FTOC. None of the dish-adherent population, dish-nonadherent population, or purified B cells alone were able to induce clonal deletion, whereas the addition of purified B cells to adherent cells restored tolerance inducibility. It was further shown that a combination of CBA/J B cells and C3H/He dendritic cells was effective in eliminating Mls-reactive clones. These results indicate that for the deletion of clones reactive with Mls antigens during T cell development in the thymus, both DC and B cells are required.

T cell tolerance to major self antigens is acquired in the thymus during T cell development. It has been shown that the clones reactive to minor lymphocyte-stimulating (Mls)1 antigens or to I-E antigens are deleted in the thymus of mouse strains that express these determinants (1–4). Studies on the cells responsible for tolerance induction in the thymus have been carried out with bone marrow chimera and thymus transplantation techniques, and it has been shown that bone marrow–derived cells play a crucial role in clonal deletion (5–7). In these experimental systems, however, it is difficult to determine which type(s) of cells among bone marrow–derived cells is responsible for clonal elimination. To identify cells mediating tolerance and to clarify the role played by such cells, it is necessary to develop an in vitro experimental system offering an environment in which T cell development occurs without negative selection. Once such a system is established, the effect of any cell population on tolerance induction can be analyzed by simply adding the candidate cell population(s) to the system.

A deoxyguanosine-treated fetal thymus organ culture (dGuo-FTOC) has been shown to provide a minimal environment for T cell development (8–10). We have recently devised a method investigating the development of T cells by injecting T cell precursors into dGuo-FTOC with a microinjector (micro i.t. method) (10). The advantages of this method are that any cell population(s) in addition to T cell precursors can be inoculated, and that the number of cells to be transferred can be exactly controlled. To identify cells responsible for clonal elimination, various cell populations from CBA/J (Mls-1') mice were injected into the dGuo-FTOC of C3H/He (Mls-1b) mice, followed by an injection of fetal thymus cells from C3H/He mice as T cell precursors. The T cells that developed in the thymus lobes were examined for Vß6 expression, and the results indicated that both dendritic cells (DC) and B cells were needed to eliminate Mls-reactive clones.

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1 Abbreviations used in this paper: DC, dendritic cells; dGuo, deoxyguanosine; FTOC, fetal thymus organ culture; micro i.t. method, the method to transfer cells into FTOC with a microinjector; Mls, minor lymphocyte-stimulating; Mφ, macrophages.
Materials and Methods

Mice. C3H/He (H-2b, Ms-1\(^{a}\), Thy-1.2), C57BL/6 (H-2\(^{b}\), Ms-1\(^{a}\), Thy-1.2), and AKR (H-2\(^{b}\), Ms-1\(^{a}\), Thy-1.1) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). CBA/J (H-2\(^{b}\), Ms-1\(^{a}\), Thy-1.2) mice were purchased from Charles River Japan Inc. (Atsugi, Japan). They were bred in our animal facility for the generation of timed pregnancies. The day of vaginal plugging was designated as day 0 of gestation. B10.Thy-1 (H-2\(^{b}\), Ms-1\(^{a}\), Thy-1.1) mice (11) were maintained in our laboratory.

Medium. RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with t-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM) (Gibco Laboratories), 2-ME (5 \( \times \) 10\(^{-5}\) M), streptomycin (100 \( \mu\)g/ml), and penicillin (100 U/ml) was used as a complete medium. FCS was purchased from Wittaker M. A. Bioproducts (Walkersville, MD).

Antibodies. Ascites fluid of hybridoma HO-13 (anti-Thy-1.2) and culture supernatants of hybridomas RL-172.4 (anti-CD4) (12) and 3.155 (anti-CD8) were used to deplete T cells. Culture supernatants of 44-22-1 (anti-V\(\beta\)6) (13), K16 (anti-V\(\beta\)8.1 and V\(\beta\)8.2) (14), and H57-597 (anti-TCR\(\alpha/\beta\)) (15) were prepared for immunofluorescence. As the second antibody, PE-conjugated anti-rat Ig (Biomeda, Foster City, CA) or FITC-conjugated anti-hamster Ig (Caltag, San Francisco, CA) were used. PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 were purchased from Becton Dickinson & Co. (Mountain View, CA).

Cell Preparations. Fetal thymus cells were obtained from day 15 fetuses of C3H/He and B10.Thy-1.1 mice, as described previously (10). Young adult thymus cells were obtained from 6-wk-old C3H/He and CBA/j mice. To prepare a Thy-1\(^{+}\) population of thymus cells, thymocytes were incubated with anti-Thy-1.2 antibody, washed, and treated twice with an appropriate dilution of rabbit complement (Cederlane, Hornby, Ontario, Canada). Spleen cells were prepared from 6-10-wk-old mice. The T cell–depleted population of spleen cells was obtained as follows. Spleen cells were incubated with anti-Thy-1.2 antibody, washed twice with culture medium, and treated twice with a mixture of anti-CD4, anti-CD8, and rabbit complement. Contamination by CD4\(^{+}\) or CD8\(^{+}\) cells in this T cell–depleted population was <1%. The T-depleted cells were passed through a Sephadex-G10 column (Pharmacia, Uppsala, Sweden) and used as purified B cells. More than 99.5% of these cells were surface Ig positive. Plastic-nonadherent cells were also obtained from the T-depleted population. The cells were suspended in complete medium with 5% FCS and cultured for 3 h in plastic dishes (25020; Corning Glass Works, Corning, NY). The floating cells were collected and used as T-depleted, dish-nonadherent cells.

Dish-adherent cells, macrophages (M\(\phi\)), and DC were obtained as described by Steinman et al. (16) with slight modifications. Briefly, a cell suspension was prepared from collagenase (Sigma Chemical Co., St. Louis, MO)-digested spleen, and low-density cells were isolated on 50% Percoll (Pharmacia). The cells were resuspended in complete medium with 5% FCS, and then allowed to adhere to plastic dishes (25020; Corning Glass Works) by culturing them for 3 h. Nonadherent cells were removed by gentle pipetting, and adherent cells were recovered by treatment with 6 mM EDTA in PBS. Contamination by lymphoid cells in the adherent populations was <3%. The adherent cells were further cultured overnight without EDTA treatment. The cells eluted from the surface and those remaining adherent were separated and used as DC and M\(\phi\), respectively. The former were >85% DC, while the latter were >90% M\(\phi\). This estimation is based on morphological and phenotypical criteria, i.e., expression of MHC class I and class II antigens and T and B cell markers (16).

Results

T Cell Development from Fetal Thymus Cells Injected into dGuo-FTOC. To determine the origin of T cells developed in dGuo-FTOC injected with fetal thymocytes, thymus cells from day 15 fetuses of AKR (Thy-1.1) mice were injected into the dGuo-FTOC of C3H/He (Thy-1.2) mice, and cultured for 14 d. The Thy-1 phenotype of the recovered cells was analyzed with a FACScan. As shown in Fig. 1, A and B, the Thy-1\(^{+}\) cells developed in the FTOC were exclusively of donor origin (Thy-1.1\(^{+}\)), confirming our previous results obtained with a different combination of mouse strains (10). Virtually no viable lymphoid cells were recovered from the dGuo-FTOC that had not been injected with fetal thymus cells (data not shown).

The next experiment was carried out to investigate the differentiation and maturation of T cells in dGuo-FTOC from inoculated fetal thymus cells. In this experiment, fetal thymus cells from C3H/He mice were injected into dGuo-FTOC of the same strain and cultured for 8 or 14 d. The mean number of recovered lymphoid cells was 4.3 \( \times \) 10\(^{4}\) per lobe on day 8 and 3.0 \( \times \) 10\(^{4}\) on day 14. Two-color flowcytometric analysis of the expression of CD4 vs CD8 is shown in Fig. 1, C and D. The results indicate that CD4\(^{+}\)8\(^{-}\) and CD4\(^{-}\)8\(^{+}\) cells (single-positive cells) as well as CD4\(^{+}\)8\(^{+}\) cells (double-positive cells) are generated by day 8. The proportion of single-positive cells increased during the culture period. This may be due to the impossibility of single-positive cells to emigrate out of the lobes or to the absence of a resupply of T cell precursors, or both. The higher proportion of mature
T cells on day 14 made it easy to determine the proportions of Vβ6high cells that are included in single-positive cells (18). Fig. 1E shows that ~3.9% of the recovered cells expressed Vβ6 at high levels.

Inability of dGuo-treated Lobes to Induce Clonal Deletion. To determine whether the thymic epithelial cells are responsible for clonal deletion, thymocytes from day 15 fetal C3H/He mice were injected into dGuo-FTOC of CBA/J or C3H/He mice, and 14 d later, the cells in the lobes were harvested for analysis. Cell recovery and CD4 vs. CD8 profiles were similar in these two groups (data not shown). Fig. 2 shows that the percentage of Vβ6high cells in T cells that developed in CBA/J lobes was virtually the same as that in T cells developed in C3H/He lobes. These results indicate that the dGuo-resistant components of fetal thymus, which are exclusively epithelial cells (8, 19, 20), are unable to induce clonal deletion.

Effectiveness of Thymic Thy-1- Cells in Clonal Deletion. Since the elimination of autoreactive T cell clones occurs predominantly in the thymus (21-23), the following experiment was carried out to examine the "tolerizing" activity of thymic non-T cells in our micro i.t. system. Adult thymus cells were treated with anti-Thy-1 plus complement to deplete mature and immature T cells as well as their immediate precursors. Although this Thy-1- population includes early T cell precursors, it takes >16 d until mature T cells are generated in the micro i.t. system (Gyotoku, J.-I. and Y. Katsura, manuscript in preparation). However, to discriminate the T cells

Figure 1. Surface phenotype of cells recovered from dGuo-FTOC injected with fetal thymus cells. (A and B) Fetal thymus cells obtained from day 15 fetuses of AKR (H-2k, Thy-1.1) mice were injected into dGuo-FTOC of C3H/He (H-2k, Thy-1.2) mice (103 cells/lobe) and cultured for 14 d. Recovered cells were stained with FITC-anti-Thy-1.1 (A) or FITC-anti-Thy-1.2 (B) and analyzed with a FACScan. The percentage of cells staining with each antibody is shown. (C-E) Fetal thymus cells from day 15 fetuses of C3H/He (H-2k, Mls-1a) mice were injected into dGuo-FTOC of C3H/He mice and cultured for 8 d (C) or 14 d (D and E). CD4 vs. CD8 profiles (C and D) and Vβ6 expression (E) were analyzed. (F-H) As controls, CD4 vs. CD8 profiles of fresh thymocytes from day 15 fetal (F) and 6-wk-old adult (G) C3H/He mice are shown. Vβ6 expression of adult thymocytes is depicted in panel H. The percentages of cells in each quadrant (C, D, F, and G) and the percentages of Vβ6high cells (E and H) are indicated. Dotted lines in E and H represent cells nonspecifically stained with the secondary antibody (PE-anti-rat IgG) alone.

Figure 2. Inability of thymic epithelial cells to induce clonal deletion. Fetal thymus cells from day 15 fetuses of C3H/He (Mls-1a) mice were injected into dGuo-FTOC of C3H/He (A) or CBA/J (Mls-1a) (B) mice. After 14 d of cultivation, cells were recovered and indirectly stained with anti-Vβ6 (solid line). Dotted lines represent cells stained with the second antibody alone. Percentages of Vβ6high cells are shown.
derived from fetal thymus cells used as the T cell precursors from those that might be generated from the Thy-1- cells, in this experiment we used B10.Thy-1.1 mice (H-2b, Thy-1.1) as the source of T cell precursors and C57BL/6 mice (H-2b, Thy-1.2) for dGuo-FTOC. Thy-1- thymus cells (10^6 cells/lobe) from CBA/J or C3H/He mice were injected into the dGuo-FTOC of C57BL/6 mice, and 2 d later, fetal thymus cells from B10.Thy-1.1 mice were injected. After 12 d of culture, cells were recovered and assayed for differentiation markers and Vβ6 expression.

A large majority (90-95%) of recovered lymphocytes expressed Thy-1.1, whereas <1% were Thy-1.2+ (Fig. 3, A and B), and the CD4 vs. CD8 profiles were similar to those shown in Fig. 1 D (data not shown), indicating that T cell development occurred normally from B10.Thy-1.1 fetal thymus cells regardless of the presence of H-2 allogeneic Thy-1- thymus cells. The Vβ6 expression of these cells is shown in Fig. 3, C and D. The proportion of Vβ6hi cells was drastically reduced in cells recovered from lobes given CBA/J Thy-1- thymus cells, indicating that thymic Thy-1- population includes cells responsible for clonal elimination.

On the other hand, a substantial proportion of cells seemed to be very weakly stained by anti-Vβ6 in the group in which Vβ6hi cells were eliminated. The staining, however, may be nonspecific since the fluorescence intensity is much lower than that of Vβ6 low-positive cells (Fig. 1 H), and since a similar staining pattern is also seen in other experimental groups regardless of the elimination of Vβ6hi cells (see other figures).

A Small Number of Splenic Non-T Cells Cause Clonal Deletion. We next investigated the capacity of splenic cells for T cell clonal deletion, because it is known that T cell tolerance is acquired not only in the thymus but also in peripheral tissues (24, 25). Spleen cells from CBA/J or C3H/He mice were depleted of T cells as described in Materials and Methods, and these cells were injected (500 cells/lobe) into the dGuo-FTOC of C3H/He mice. 2 d later, these lobes were injected with C3H/He fetal thymus cells, and the cells were harvested 14 d later for analysis. The CD4 vs. CD8 profiles of the recovered cells were similar in these two groups (not shown). Fig. 4, A and B shows that the proportion of cells expressing TCR-α/β does not differ between these two groups. The percentage of Vβ6hi cells among the cells recovered from lobes given C3H/He cells was 3.8%, while only 0.8% of the cells from those given CBA/J cells were Vβ6hi (Fig. 4, C and D). These results indicate that T-depleted spleen cells of CBA/J mice are effective in specifically eliminating Vβ6+ clones.

Failure of Splenic Adherent Cells, Nonadherent Cells, or Purified B Cells to Induce Clonal Deletion. Since splenic non-T cells comprise various types of cells, we tried to determine which type(s) of cells were responsible for clonal deletion. The cells were divided into dish-adherent and nonadherent populations. The adherent cells include Mφ and DC, whereas the nonadherent cells are supposed to be mainly B cells. The ex-
Experimental protocol is the same as that described in the preceding section. Vβ6 expression of cells recovered from lobes given adherent cells and nonadherent cells is shown in Fig. 5, A–D. The results indicate that neither adherent cells nor nonadherent cells alone of CBA/J mice are effective in eliminating Vβ6+ clones during T cell development.

It is well known that the Mls antigen is predominantly expressed on B cells (26, 27). Although splenic nonadherent cells, which are mainly B cells, were unable to induce clonal deletion, the possibility exists that highly purified B cells can induce the deletion. Results of the experiment to examine this possibility are shown in Fig. 5, E and F. It was found that B cells from Mls-1+ mice were unable to eliminate Vβ6+ cells. Approximately 7% of cells harvested from the FTOC were surface IgM+ (data not shown), indicating that the B cells injected into the lobes survived and even increased in number in the thymic lobes. Thus, the inability of B cells to induce T cell clonal deletion cannot be ascribed to the death of B cells. These results collectively suggest that at least two distinct cell populations, one included in adherent cells and the other included in nonadherent cells, are required for clonal deletion of Vβ6+ T cells to occur.

Mixture of B Cells and Adherent Cells Was Able to Induce Clonal Deletion. dGuo-FTOC of C3H/He mice were injected with a 1:1 mixture of adherent cells from C3H/He mice and B cells from C3H/He or CBA/J mice. Fetal thymus cells from C3H/He mice were added 2 d later, and cells were harvested 14 d thereafter. Two-color flow cytometric analysis showed that normal T cell development occurred in both groups (data not shown). The expression of Vβ6 is shown in Fig. 6, A and B. The results indicate that a combination of Mls-1B cells (CBA/J) and Mls-1A adherent cells (C3H/He) is capable of inducing the deletion of Vβ6+B cells. In contrast, the proportion of KJ16-reactive cells, which comprised Vβ8.1+ and Vβ8.2+ cells, was only slightly affected by a combination of adherent cells and Mls-1B cells (Fig. 6, C and D). The slight reduction in the proportion of KJ16-reactive cells in Fig. 6 D may be due to the elimination of Vβ8.1+B cells, which represent another Mls-1-reactive population (2), without affecting Vβ8.2+ cells.

DC but Not Mφ Were Required in Combination with B Cells to Eliminate Vβ6+ T Cells. DC and Mφ are the major components of adherent cells. The next question to be addressed was whether they were responsible for the elimination of Vβ6+ T cells in the presence of Mls-1B cells. dGuo-FTOC of C3H/He mice were injected with a 1:1 mixture of C3H/He DC and C3H/He B cells, C3H/He DC and CBA/J B cells, C3H/He Mφ and C3H/He B cells, or...
C3H/He Mφ and CBA/J B cells. 2 d later, these lobes were injected with C3H/He fetal thymus cells, and T cells generated 14 d thereafter were analyzed for expression of Vβ6+ cells.

As shown in Fig. 7, clonal deletion was observed only in the group receiving DC and CBA/J B cells (B). Administration of Mφ and CBA/J B cells was not effective in eliminating, but even slightly enhanced, the generation of Vβ6+ cells. These results show that it was DC that were required to induce clonal elimination in the presence of Mls-expressing B cells.

Discussion

The present study was performed to identify cells responsible for clonal elimination of Mls-reactive T cells during T cell development in the thymus. The experiments were carried out by taking advantage of a micro i.t. transfer system (10), which makes it possible to inoculate arbitrary populations of cells in desired numbers into dGuo-FTOC, where T cell development occurs without negative selection if "tolerizing" cells are not transferred. With this experimental system, it was strongly suggested that the cooperation of DC and Mls-expressing B cells was required for the elimination of Mls-reactive T cell clones.

The importance of bone marrow-derived cells has been demonstrated in intrathymic tolerance to class I (28, 29) and class II (30) MHC antigens. Recently, it was shown that the expression of I-E molecules on bone marrow-derived cells was a prerequisite for clonal elimination of Vβ6+ (6, 7, 31) and Vβ17a+ (5) T cells. Moreover, the clonal deletion could be induced in irradiated Mls-1b mice, which received a mixture of bone marrow cells from I-E+' Mls-1b and I-E-' Mls-1b mice (4, 31), indicating that I-E and Mls molecules do not necessarily need to be expressed on the same cells. The results also suggest that two distinct types of cells cooperate in eliminating Mls-reactive clones. These findings are compatible with our present finding, assuming that the former and the latter represent DC and B cells, respectively.

The effectiveness of DC in the induction of intrathymic tolerance to class I antigens was shown by Matzinger and Guerder (32) in their experiments carried out in an organ culture system. However, it was not clarified whether or not other types of cells residing in the FTOC were required to cooperate with DC, since the FTOC they used were not treated with dGuo. It is probable, however, that class I tolerance is induced by DC alone because DC express class I antigens on the surface (16, 33). The requirement of B cells could be restricted to such antigens as Mls, which are not expressed by DC but are predominantly expressed by B cells (26, 27). Since it seems unlikely that DC express all self antigens, some of the self antigens should be transferred to DC from other types of cells. The present study may provide an example of how intrathymic clonal deletion is induced in the case of self antigens that are mainly expressed in the periphery.

DC have been shown to be the most effective APC for priming mature T cells (34, 35). Although B cell lines or activated B cells can induce a proliferative response of Mls-reactive T cells, normal resting B cells only poorly stimulate T cells without the help of adherent cells (26, 27). Since the DC used for tolerance induction in the present study, as well as in other studies (32), are derived from the spleen, it is probable that immature T cells may die by receiving the same signal from DC that otherwise primes mature T cells. In the thymus, DC are known to be distributed mainly in the cortico-medullary junction (36), the area where the T cells of intermediate stages between immature double-positive cells and mature single-positive cells are believed to reside (37, 38). Because such stages of T cells are suggested to be feasible for clonal elimination (39–41), and the clonal elimination of Vβ6+ T cells has actually been shown to occur in this area (21), it is highly probable that the major function of DC in the thymus is to "tolerize" the autoreactive clones.

The present study does not support the possibility that Mφ play some role in clonal deletion. The failure of Mφ to function in clonal elimination may be ascribed to their inability to stimulate normal T cells (35), although they have been shown to help the stimulating activity of DC (42, 43). On the other hand, we have recently shown that Mφ are effective in promoting T cell development in the thymus (Gyotoku et al., manuscript in preparation). Thus, Mφ and DC in the thymus appear to play different roles in T cell development.

The role of B cells in the clonal deletion of Mls-reactive T cells may be to provide Mls molecules to DC (Figs. 6 and 7), though the possibility has not been definitively excluded that B cells play some direct functional role. Consistent with
this notion is our unpublished finding that Vβ6+ T cells are not deleted in CBA/N mice. Although CBA/N mice carry the Mls-1' allele, their spleen cells have been shown to be deficient in Mls-stimulatory activity (44). Since the xid mutation in CBA/N mice is expressed as a B cell dysfunction, it is probable that the failure of the spleen cells to stimulate Mls-reactive T cells is due to the lack of expression of Mls antigens on B cells, which consequently may have resulted in the failure to delete Vβ6+ T cells.

In normal situations, clonal deletion does not occur until ~1 wk after birth (45, 46). This may be due to the insufficiency in number or the functional immaturity of DC and/or B cells in the thymus at fetal and newborn ages, since these cells have been shown to exist in the fetal thymus (our unpublished data). On the other hand, the origin of DC and B cells in the thymus has not been determined. Although multipotent stem cells exist in both the fetal and adult thymus (47; Gyotoku, J.-I., and Y. Katsura, manuscript in preparation), it is unclear whether DC are generated from these stem cells. In contrast, donor-type B cells are observed in dGuo-Mls-reactive T cells (Ideyama, S., and Y. Katsura, unpublished data). However, it is not certain whether these B cells are derived from intrathymic stem cells. It is possible that these cells are not necessarily produced in the thymus but migrate into the thymus after they have matured outside the thymus. If the latter is true, it is tempting to speculate that some self antigens are trapped by these cells and brought into the thymus to induce self tolerance.

Recent studies have shown that tolerance is not necessarily due to clonal elimination but may also be attributed to clonal anergy (48–53). Since T cell anergy can be induced in various conditions, it is difficult to discuss the mechanisms of clonal elimination with reference to those of anergy. Of interest, however, is that the anergy state is observed in radiation chimera where the I-E antigen is selectively expressed on thymic epithelial cells (50, 52). These findings suggest that the developing T cells do not die but fall into anergy if the signal(s) given at an immature stage is “incomplete". In this context, we plan to determine whether anergy is induced in our experimental system in which DC are absent but the Mls antigen is expressed on epithelial cells, Mφ, or B cells.

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