T Cell Receptor–mediated Signaling Events in CD4+CD8+ Thymocytes Undergoing Thymic Selection: Requirement of Calcineurin Activation for Thymic Positive Selection but Not Negative Selection

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Summary

The goal of this study was to identify the differences of intracellular signals between the processes of thymic positive and negative selection. The activation of calcineurin, a calcium- and calmodulin-dependent phosphatase, is known to be an essential event in T cell activation via the T cell receptor (TCR). The effect of FK506, an inhibitor of calcineurin activation, on positive and negative selection in CD4+CD8+ double positive (DP) thymocytes was examined in normal mice and in a TCR transgenic mouse model. In vivo FK506 treatment blocked the generation of mature $TCR^{high}CD4+CD8^-$ and $TCR^{high}CD4^-CD8+$ thymocytes, and the induction of CD69 expression on DP thymocytes. In addition, the shutdown of recombination activating gene 1 (RAG-1) transcription and the downregulation of CD4 and CD8 expression were inhibited by FK506 treatment suggesting that the activation of calcineurin is required for the first step (or the very early intracellular signaling events) of TCR-mediated positive selection of DP thymocytes. In contrast, FK506-sensitive calcineurin activation did not appear to be required for negative selection based on the observations that negative selection of $TCR\alpha\beta$ T cells in the $H-2^b$ male thymus (a negative selecting environment) was not inhibited by in vivo treatment with FK506 and that there was no rescue of the endogenous superantigen-mediated clonal deletion of $V\beta6$ and $V\beta11$ thymocytes in FK506-treated CBA/J mice. DNA fragmentation induced by TCR activation of DP thymocytes in vitro was not affected by FK506. In addition, different effects of FK506 from Cyclosporin A on the T cell development in the thymus were demonstrated. The results of this study suggest that different signaling pathways work in positive and negative selection and that there is a differential dependence on calcineurin activation in the selection processes.

The developmental fate of individual T cells maturing in the thymus is determined by the specificity of the TCRs they express. The maturation of thymocytes expressing TCRs with potential reactivity with self-ligands is aborted by a process termed negative selection (1–5), whereas the maturation of thymocytes expressing TCRs that are potentially reactive with foreign antigens presented by self-MHC–encoded molecules is promoted by a process called positive selection (6–10). Because intrathymic selection events are clonally restricted and TCR specific, they are thought to be a consequence of TCR-mediated signals stimulated by interaction with thymic stromal cells bearing self-ligands. We have been investigating the nature of events in the TCR-mediated selection process in the thymus.

Recently, we demonstrated that thymocytes undergoing positive selection can be identified on the basis of the expression of an "early activation marker," CD69, on their surface (11). Analysis of other surface markers in combination with CD69 revealed that the CD69-positive thymocytes consisted of several distinct populations based on surface phenotypes, suggesting that positive selection involves multi-step processes. The first population identified appeared as $CD69^{low}TCR^{low}CD4^{low}CD8^{low}$ cells of the CD4+CD8+ double positive (DP)[1] thymocyte subpopulation (11). The next phenotypic

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[1] Abbreviations used in this paper: APC, allophycocyanin; $[Ca^{2+}]_i$, intracellular calcium; DN, double negative; DP, double positive; EtBr, ethidium bromide; FCM, flow cytometry; H-YTg, anti-H-Y $TCR\alpha\beta$ transgenic; PI, propidium iodide; RAG-1, recombination activating gene 1; SEB, staphylococcal enterotoxin B; SP, single positive.

change was the concomitant increase in surface expression of CD69 and TCR. Further development to CD4 or CD8 single positive (SP) thymocytes began just after the increase of TCR and CD69 expression, and resulted in the appearance of either CD4^{high}CD8^{low} or CD4^{low}CD8^{high} cells. These cells apparently were destined to become CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ SP thymocytes, respectively. Finally, the SP thymocytes lost the expression of CD69 and heat stable antigen, acquired Qa-2, and became very mature SP thymocytes in many criteria (11–14).

In support of our hypothesis that positive selection involves multi-step processes, several investigators have recently reported that the thymic positive selection consisted of at least two distinct stages (15–19). T cell development up to CD4^{high} CD8^{low} or CD4^{low}CD8^{high} DP thymocyte stages was mediated by CD4- and CD8-independent signals (stochastic events) (15–19), and further development to mature CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ SP thymocyte stages was mediated by signals dependent on CD4 and CD8 molecules, because no mature CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ SP thymocytes were generated in MHC class II or I knockout mice, respectively.

Although the molecular mechanisms in each step of the positive selection have not been fully resolved, there are emerging views from recent published reports. When the TCR on DP thymocytes were engaged with anti-TCR mAb in vitro, transcription of recombination activating gene 1 (RAG-1) and RAG-2 in DP thymocytes was turned off (20, 21), expression of CD4 and CD8 was downregulated (11, 22), and surface expression of CD69 was induced (11). These changes appeared to take place in the first step of thymic positive selection within the DP thymocyte population, because the first distinct population observed during positive selection was comprised of CD69-positive CD4^{low}CD8^{low} DP thymocytes. We wished to gain a better understanding of the intracellular signaling events in the first step of positive selection.

The effect of the immunosuppressive drug, Cyclosporin A (CsA), on T cell development in the thymus has been investigated by several groups (23–25). CsA and FK506 are known to bind immunophilins (cyclophilin and FKBP, respectively) (reviewed in 26) and have been shown to inhibit the activation of calcineurin, a calcium- and calmodulin-dependent phosphatase, resulting in the inhibition of T cell activation. In vivo treatment of mice with CsA was shown to inhibit the generation of SP thymocytes (23–25). The mechanism by which CsA blocks this process has not been fully investigated. It was of interest to determine whether the blockade of SP thymocyte generation could be due to interfering signals occurring in the positive selection process of the DP thymocyte population. We were also interested in determining whether the effect of FK506 on T cell development might differ from that of CsA, since the pharmacology of FK506 and CsA is quite different (26).

In this study, we have examined the effect of FK506 on thymic positive and negative selection in several different systems that involve the use of normal mice and a H-Y-specific TCRαβ transgenic mouse model. The results show that

FK506 blocked the generation of mature SP thymocytes by inhibiting the first step of positive selection in the DP thymocyte population. In contrast, FK506 treatment had no effect on negative selection events.

Materials and Methods

Animals. C57BL/6 (B6) young adult mice and B6 and CBA/J pregnant females were purchased from Japan SLC Inc. (Shizuoka, Japan). Anti-H-Y TCRαβ transgenic (H-YTg) mice (5) and Vβ8.2 TCR transgenic (Vβ8Tg) mice (27) were established by Drs. H. von Boehmer and M. Steinmetz (Basel Institute, Basel, Switzerland), and provided by Dr. Alfred Singer (National Cancer Institute, Bethesda, MD). All mice used in this study were maintained under specific pathogen-free conditions.

Reagents and Cell Lines. A hamster anti–mouse CD69 mAb (H1.2F3) was kindly provided by Dr. E. M. Shevach (National Institutes of Health, Bethesda, MD) (28). FITC-conjugated anti-CD69 (H1.2F3-FITC) and biotinylated anti-CD69 (H1.2F3-biotin), anti-TCRαβ-FITC (H57-597-FITC) (29), allophycocyanin (APC)-conjugated anti-CD8 (53-6.72-APC) (30), anti-TCRα of H-YTg TCR mAb-FITC (T3.70-FITC) (31) and anti-TCRα of 2B4 T cell hybridoma mAb-FITC (A2B4-FITC, as a negative control) (32) were prepared in our laboratory (University of Tokyo). PE-conjugated anti-CD4 mAb (GK1.5-PE) was purchased from Becton Dickinson & Co. (Mountain View, CA). PE-conjugated streptoavidin (PE-avidin) was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Anti-TCRVβ6 mAb (44-22-1) (33), anti-TCRVβ11 mAb (RR3-15) (34), and anti-TCRVβ8.2 mAb (F23.2) (35) were used as culture supernatants. Goat anti–mouse IgG-FITC and goat anti–rat IgG-FITC were purchased from Southern Biotechnology Associates (Birmingham, AL).

Cell lines used in this study were the FcR$^+$ B cell line LK35.2 (36), I-E^k transfected L cell line DCEK (37), and the T cell hybridoma line 68-41 (38).

Immunofluorescent Staining and Flow Cytometry Analysis. Freshly prepared thymocytes from FK506-treated mice and in vitro cultured thymocytes were suspended in PBS supplemented with 2% FCS and 0.1% sodium azide. In general, one million cells were incubated on ice for 30 min with the appropriate staining reagents using a standard method as previously described (39). For multicolor flow cytometry (FCM) analyses, electronic compensation was performed using cell mixtures of positive and negative cell populations in each fluorescence emission. FCS analysis was performed on FACStar Plus®, using FACStar Plus® and Consort 30 software programs (Becton Dickinson & Co.) for data collection and analysis. Fluorescence data were collected as a list mode on 20,000 viable cells as determined by light scatter parameters and propidium iodide (PI) exclusion.

In Vivo Treatment with FK506 and CsA. Neonatal B6, CBA/J, or H-YTg mice were injected intraperitoneally daily with FK506 (Fujisawa Pharmaceutical Co., LTD, Osaka, Japan) or CsA (Sandozpharma, Basel, Switzerland) for 7 or 14 d beginning at day 0 (within 24 h). FK506 was suspended in PBS and CsA in olive oil. 20 h after the last injection, thymocytes were prepared from individual thymi and subjected to three-color FCM analysis.

Purification of CD4$^+$CD8$^+$ (DP) Thymocytes. DP thymocytes were isolated by adherence to plates coated with anti-CD8 mAb (83-12-5) (40), and were >96% CD4$^+$CD8$^+$ as described (41).

In Vitro Stimulation of Thymocytes with Immobilized anti-TCR mAb. B6 whole thymocytes or purified DP thymocytes were stimulated in vitro with immobilized anti-TCR mAb (H57-597) for the times indicated at 37°C in 24-well culture plates (model

3424; Costart Corp., Cambridge, MA) in 2 ml of RPMI 1640 (GIBCO BRL, Gaithersburg, MD) culture medium, supplemented with 10% (vol/vol) FCS, 1 mM L-glutamine, antibiotics (penicillin and streptomycin), and 5 × 10⁻⁵ M 2-mercaptoethanol. The 24-well culture plates were preincubated for 1 h at 37°C with 100 μg/ml of H57-597 and then washed with RPMI 1640 culture medium before adding cell suspension. Viability of thymocytes at the end of the stimulation culture was determined to be >85% by three criteria: trypan blue exclusion, PI exclusion, and forward light scatter.

Northern Blot Analysis. Total RNA was prepared by the acid phenol extraction procedure as described (42). Total RNA (20 μg) isolated from the purified DP thymocytes stimulated with immobilized anti-TCR was separated on 1% agarose gels, and transferred to Biodyne membrane filters (Pall BioSupport, East Hills, NY). The filters were hybridized with ^{32}P-labeled mouse CD69 (43) or RAG-1 (44) probes. The mouse CD69 probe was kindly provided by Dr. S. Ziegler (Immunex Corp., Seattle, WA) and the RAG-1 probe was kindly provided by Dr. F. A. Alt (Harvard Medical School, Boston, MA). After overnight hybridization, the filters were washed twice in 2× SSC at room temperature for 15 min and then in 0.2× SSC at the same temperature for 15 min. The filters were subjected to autoradiography.

Measurement of Intracellular Calcium Concentration ([Ca²⁺]ᵢ) by Fluorescence Microscopy and Digital Image Analysis. The fluorescence digital image processing system used is called IMRAS (45) and is similar to that described by Dr. Carl H. June (46). The hardware of IMRAS consisted of a modified image processor ARGUS-100 (Hamamatsu Photonics, Hamamatsu, Japan), an inverted microscope (Nikon, Tokyo, Japan) with a stage incubator (Tabai Espec, Osaka, Japan), an image intensifier with an electronic zooming camera (Hamamatsu Photonics), and a light house producing double beams (340 ± 10 nm and 380 ± 10 nm) in a modified spectrofluorometer (model CAM220; Nippon Bunko, Tokyo, Japan) connected to the microscope. The image processor and spectrofluorometer were interfaced to a personal computer (EPSON, Tokyo, Japan). The images were averaged and the background was subtracted (46, 47).

Purified B6 DP thymocytes (10⁷) were precultured in single cell suspensions at 37°C for 2 h, and then washed in Hanks' solution supplemented with 0.1% FCS (Fura-2 medium). The cells were then loaded with 4.4 μM Fura-2, AM (Molecular Probes, Inc., Eugene, OR) at 37°C for 30 min (46). They were washed three times with Fura-2 medium, incubated at 37°C for 5 min, and then placed on a glass slide in a reaction chamber on the stage of an inverted microscope. The Fura-2–loaded responder thymocytes (10,000 cells) were overlaid with an excess number (100,000) of unloaded FcR⁺ LK35.2 cells. After the chamber was filled with 1 ml of 37°C Fura-2 medium, measurement of [Ca²⁺]ᵢ in the responder thymocytes was started by using a two wavelength (340 and 380 nm) method as described (46). [Ca²⁺]ᵢ was monitored every 15 s for 8 min at 37°C. Stimulation of T cells was done by adding anti-CD3 mAb (145-2C11, 50 μg/ml) (48) to the chamber 1 min after starting the measurement. Temperature of the medium in the reaction chamber was controlled at 37°C throughout the measurement by a thermostat.

Immunoblotting. Immunoblottings with anticalcineurin antibodies were done by the method described (39, 41). In brief, thymocytes from male or female H-YTg mice were lysed in 1% NP-40 containing lysis buffer (39). The lysates and purified bovine calcineurin as a control (#14-104; Upstate Biotechnology, Inc., Lake Placid, NY) were subjected to 12.5% SDS-PAGE under reducing conditions and electrotransferred to a nylon membrane. The mem-

branes were then immunoblotted with polyclonal anticalcineurin antibody (#06-144) or a monoclonal anticalcineurin β antibody (#05-187). These antibodies were purchased from Upstate Biotechnology, Inc. A chemoluminescence detection system (ECL; Amersham International, Amersham, Bucks, UK) was used for visualization. The band intensities were measured by a densitometer, and an arbitrary densitometric unit was listed under each band.

DNA Fragmentation and Thymocyte Death Assay. B6 or Vβ8Tg DP thymocytes (10⁷) were first precultured in a single cell suspension in 24-well culture plates at 37°C for 2 h. The precultured DP thymocytes (10⁷) were then stimulated with anti-TCR mAb and FcR⁺ LK35.2 cells (10⁶), or with staphylococcal enterotoxin B (SEB) (10 μg/ml) and I-E⁺ transfected L cells, DCEK, as described (49). 68-41 T hybridoma cells (10⁶) were stimulated with immobilized anti-TCR mAb (H57-597) for 24 h. FK506 (10 nM) or CsA (100 nM) was added at the beginning of precultures. Genomic DNA from the stimulated cells was separated as described (49). The DNA samples were run on 0.8 or 2% agarose gels containing 0.5 μg/ml ethidium bromide (EtBr; Sigma Chemical Co., St. Louis, MO) at 10 V for 16 h. The gels were photographed using ultraviolet light illumination.

For the thymocyte death assay using EtBr (50, 51), B6 thymocytes were stimulated as above and harvested. Where indicated, FK506 (10 nM), CsA (100 nM), EGTA (5 mM), EDTA (5 mM), hydrocortisone (1 μM), or thapsigargin (15–150 nM; Sigma Chemical Co.) was added at the beginning of precultures. The cultured cells were first stained with anti-CD4-PE and anti-CD8-APC, washed, and then stained with EtBr (1 μg/ml) for 30 min. After washing extensively, the cells were subjected to three-color FCM analysis. LK35.2 stimulator cells were excluded from FCM analysis by using forward scatter during acquisition. Percentages of EtBr⁺ cells (both EtBrⁱⁿᵗ and EtBrʰⁱᵍʰ cells) in electronically gated DP thymocytes were determined.

Results

In Vivo Treatment with FK506 Inhibits the Generation of SP Thymocytes. It has been previously reported that the generation of CD4 and CD8 SP thymocytes is inhibited by the in vivo treatment of mice with CsA (23–25). Given that both CsA and FK506 are immunosuppressive drugs with similar modes of action, it was of importance to determine whether FK506 would have a similar effect on the generation of SP thymocytes in view of the differences in the pharmacology of these two drugs. Therefore, neonatal B6 mice (three mice per group) were treated daily for 7 d beginning at day 0 (within 24 h of birth) with 1, 3, 10, or 30 μg of FK506 in PBS, or with 3, 10, 30, or 100 μg of CsA in olive oil. 20 h after the last injection, the mice were killed and thymocytes were individually prepared and stained with anti-CD4-PE and anti-CD8-APC for FCM analysis. Yields of thymocytes, numbers of CD4⁻CD8⁻ double negative (DN) thymocytes, CD4⁺CD8⁺ DP and CD4⁺CD8⁻ (CD4 SP) thymocytes from the treated mice are shown in Fig. 1. Total numbers of thymocytes from the mice treated with 1, 3, or 10 μg of FK506 were not changed compared with those of PBS-treated mice (Fig. 1 *A*). In contrast, the mice treated with CsA showed decreased numbers of thymocytes in a dose-dependent manner. Next, the numbers of DN, DP, and CD4 SP thymocytes present in the thymus of the mice treated with FK506 and
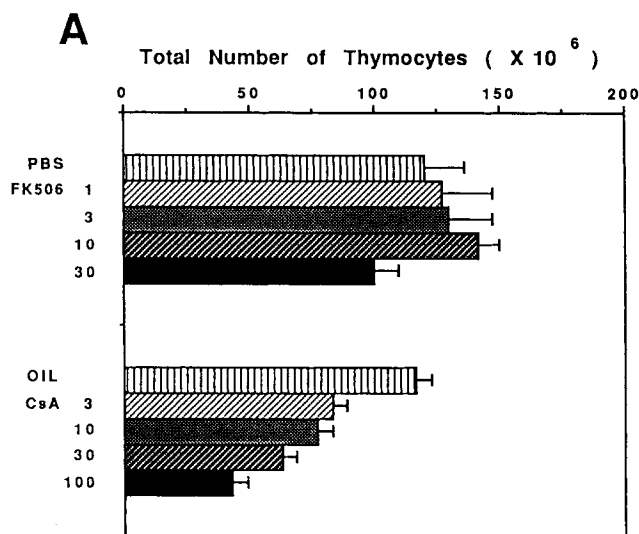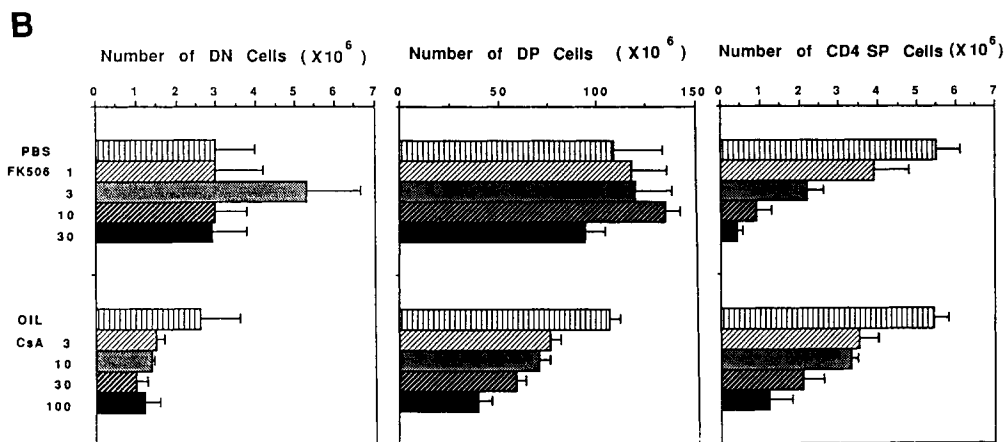
A

Total Number of Thymocytes (X 10^6)

Figure 1. Effect of in vivo treatment with FK506 and CsA on the T cell development in B6 neonatal mice. Neonatal B6 mice (three mice per group) were treated daily for 7 d beginning at day 0 (within 24 h of birth) with indicated doses of FK506 in PBS or CsA in olive oil. Thymocytes were individually prepared and stained with anti-CD4-PE and anti-CD8-APC. Yields of thymocytes (mean) are depicted with standard deviations (A). The number of CD4$^-$CD8$^-$ DN, CD4$^+$CD8$^+$ DP, and CD4$^+$CD8$^-$ (CD4 SP) thymocytes were estimated by using the number of the yield of thymocytes and the CD4/CD8 profile of each mouse (B). The mean values are shown with standard deviation.

B

Number of DN Cells (X10^6) Number of DP Cells (X10^6) Number of CD4 SP Cells (X10^6)

CsA were estimated by using the CD4 and CD8 staining profiles. As shown in Fig. 1 B, no effect of FK506 on the generation of DN and DP thymocytes and dose-dependent inhibition of the generation of CD4 SP thymocytes was detected, whereas CsA did inhibit both DP and CD4 SP thymocyte generation. These results indicate that FK506 treatment inhibits T cell development of CD4 SP from the DP stage but does not appear to affect the development of thymocytes through the DP stage. Since CsA appeared to block T cell development to the DP thymocyte stage, the decreased number of CD4 SP thymocytes detected in the CsA-treated mice could be a reflection of the inhibition of DP thymocyte generation. Thus, to evaluate the role of calcineurin activation during thymic selection events, we chose FK506 for further experiments.

The Expression of CD69 in DP Thymocytes Is Inhibited by Treatment with FK506 In Vivo. CD69, an early activation antigen expressed on ∼10% of normal thymocytes, was shown to be a marker of thymocytes undergoing TCR-mediated thymic selection (11, 52, 53). All CD69$^+$ thymocytes express TCRαβ and they include a portion of the DP thymocytes and ∼50% of the CD4 and CD8 SP thymocytes

(11). Consequently, we examined the CD69 expression on thymocytes of mice treated with FK506. The CD4/CD8 and TCRαβ/CD69 profiles of thymocytes of normal mice treated with 1 or 10 μg of FK506 are shown in Fig. 2. CD4/CD8 profiles show that the inhibition of CD4 SP thymocytes was dose dependent. Dose-dependent decreases in the numbers of CD69$^+$ cell and TCRαβ$^+$ cell populations were also noted in the FK506-treated mice. In fact, few TCRαβ T cells were observed in the 10-μg treated group, consistent with the inhibition of the generation of SP thymocytes.

The CD69 expression on DP thymocytes of the FK506-treated mice was determined by three-color FCM analysis with anti-CD4, anti-CD8, and anti-CD69 mAb. The CD69 expression on the electronically gated DP thymocytes of the FK506-treated mice are depicted in the right panels of Fig. 2. The numbers of CD69$^+$ cells in the DP thymocyte population were decreased in a dose-dependent manner in the FK506-treated mice. This result indicated that the treatment with FK506 also caused the inhibition of CD69 expression by DP thymocytes. Similar observations have been obtained in experiments using fetal thymus organ cultures in the presence of FK506 or from adult mice treated in vivo with an
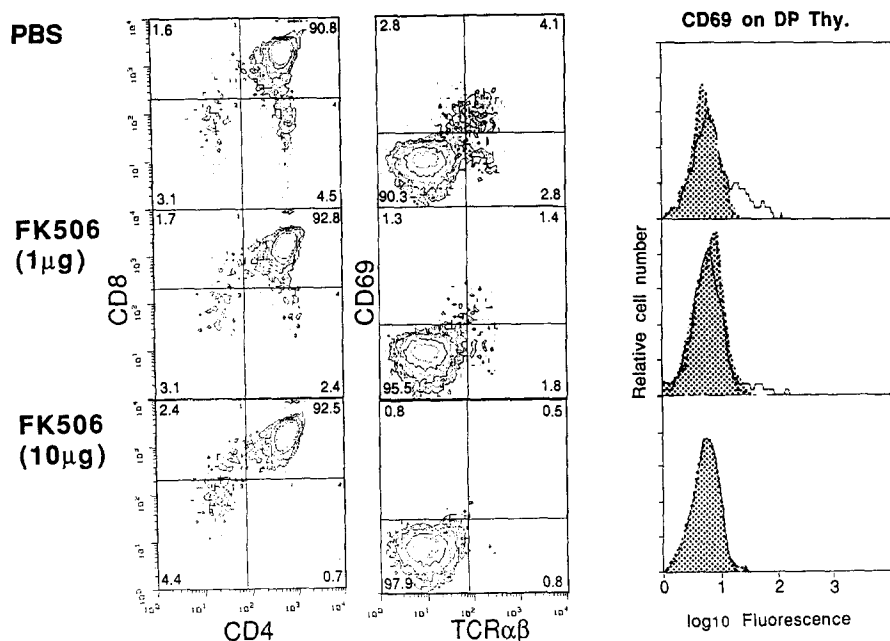
PBS

FK506 (1μg)

FK506 (10μg)

CD8

CD4

CD69

TCRαβ

CD69 on DP Thy.

Relative cell number

log10 Fluorescence

Figure 2. Effect of in vivo treatment of FK506 on T cell development in the thymus. B6 neonatal mice (five to seven mice per group) were treated daily for 7 d beginning at day 0 (within 24 h of birth) with 1 or 10 μg of FK506 in PBS. 20 h after the last injection, the mice were killed and thymocytes were individually prepared for FCM analysis with anti-CD4-PE, anti-CD8-APC, and anti-CD69-FITC, or with anti-TCR-FITC and anti-CD69-biotin followed by PE-Avidin. Representative patterns of the CD4/CD8 profiles, the TCRαβ/CD69 profiles, and the CD69 expression levels (*solid lines*) on electronically gated CD4$^+$CD8$^+$ (*DP*) thymocytes (*upper right quadrants* in the CD4/CD8 profiles) are shown with background stainings (*shaded areas*). The percentages of cells present in each area are also indicated.

equivalent dose (per body weight) of FK506 for 1 wk (data not shown). Data are not shown, however, CsA showed a similar effect on the expression of CD69 on DP thymocytes. In the case of the adult mice experiments, no toxic effect of FK506 on mature cells was noted since normal numbers of mature T cells were recovered from FK506-treated animals (data not shown).

The surface expression of CD69 on DP thymocytes in the thymus is thought to be a consequence of intracellular signaling events initiated by TCR engagement of ligands on thymic stromal cells (11, 52, 53). We have demonstrated that DP thymocytes express CD69 within a few hours after in vitro stimulation with immobilized anti-TCR mAb (11). Thus, we evaluated the effect of FK506 on the expression of CD69 in anti-TCR–activated DP thymocytes. Normal B6 thymocytes were stimulated in vitro with immobilized anti-TCR mAb (H57-597) in the presence or absence of 10 nM of FK506 for 14 h at 37°C. The addition of 10 nM of FK506 is known to inhibit completely the anti-TCR–induced proliferative response of thymocytes, mature splenic T cells, or T cell clones (data not shown). The cultured thymocytes were harvested, washed, and then stained with anti-CD4-PE, anti-CD8-APC, and anti-CD69-FITC. Dead cells were excluded from the FCM analysis by the use of PI. CD4/CD8 profiles and CD69 profiles of total, electronically gated DP, CD4SP, and CD8SP thymocytes are shown in Fig. 3 A. The induction of CD69 on DP thymocytes was found to be markedly inhibited by FK506. In addition, the expression of CD69 on mature SP thymocytes was inhibited by FK506. A similar effect of CsA was observed (data not shown).

We tested also the effect of FK506 on the CD69 induction at the mRNA level. Purified DP thymocytes were stimulated with immobilized anti-TCR mAb in the presence of 10 nM of FK506 for 0.5 and 1.0 h. Northern blot analysis was performed using a probe for mouse CD69. As shown in Fig.

3 B, CD69 mRNA was induced within one-half hour in DP thymocytes after anti-TCR stimulation, and this induction was inhibited significantly but not completely by the addition of FK506. Therefore, we conclude on the basis of these collective results that the expression of CD69 in DP thymocytes is partially dependent on the FK506-sensitive calcineurin activation.

The Effect of FK506 on the Downregulation of CD4 and CD8 Expression and the Shutdown of RAG-1 Transcription in DP Thymocytes after Anti-TCR Stimulation In Vitro. The treatment of DP thymocytes in vitro with anti-TCR mAb has been shown to also result in the downregulation of CD4 and CD8 expression (11, 22) and the shutdown of the transcription of the RAG-1 and RAG-2 genes (20, 21). As these events occur in the early steps of thymic positive selection, we investigated the effect of FK506 treatment on these events. The CD4^{low} CD8^{low} phenotype is characteristic of the first distinct population of thymocytes undergoing positive selection, i.e., CD69$^+$ CD4^{low}CD8^{low} DP thymocytes (11). As shown in Fig. 4, increased numbers of CD4^{low}CD8^{low} DP thymocytes are observed in the anti-TCR mAb–treated cultures of purified DP thymocytes. Since these cells were not stained by PI and trypan blue, and survived for more than 48 h (data not shown), they did not appear to be under the negative selection process. This phenotypic change was observed also in the unseparated thymocytes stimulated with anti-TCR mAb (*arrow*, Fig. 3, CD4/CD8 profiles). However, in the FK506-treated cultures, no increase in thymocytes with this phenotype was detected. Yields of viable thymocytes were similar in the range of >90% of the input number of thymocytes. Thus, it would appear that the downregulation of CD4 and CD8 expression on DP thymocytes, which occurs as a consequence of TCR-mediated signaling events, involves calcineurin activation.

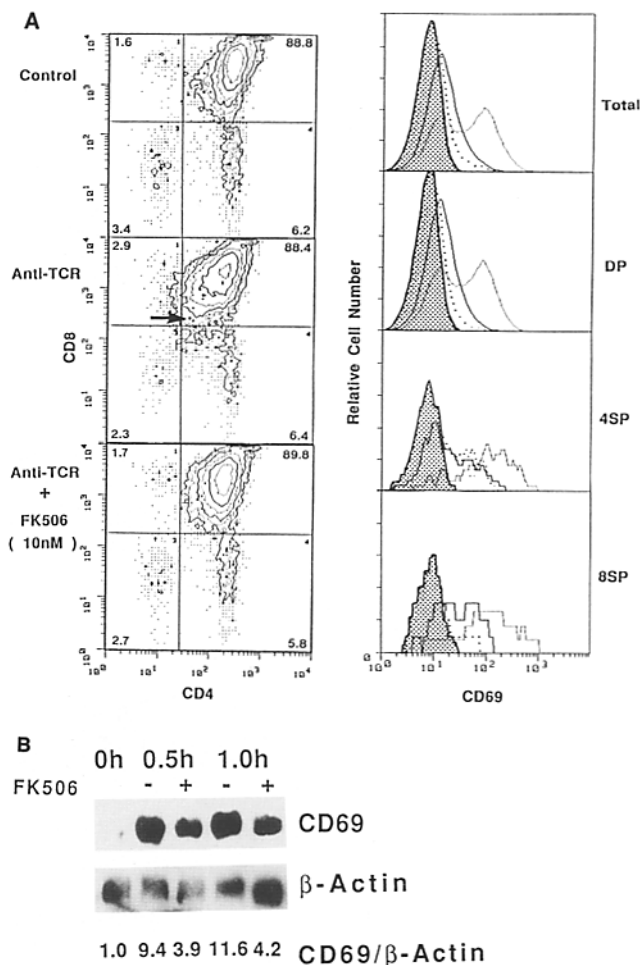Next, we determined the effect of FK506 treatment on

A

B

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        0h    0.5h   1.0h
FK506   -  +   -  +
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CD69

β-Actin

1.0 9.4 3.9 11.6 4.2   CD69/β-Actin

Figure 3. Effect of FK506 on the cell surface expression and transcription of CD69 in DP thymocytes stimulated with anti-TCR mAb in vitro. (*A*) Normal B6 thymocytes were stimulated in vitro with immobilized anti-TCR mAb (H57-597) in the presence or absence of 10 nM of FK506 for 14 h at 37°C. The cultured thymocytes were washed and then subjected to three-color FCM analysis with anti-CD4-PE, anti-CD8-APC, and anti-CD69-FITC. Dead cells were excluded from the FCM analysis using PI. CD4/CD8 profiles (*left*) and CD69 profiles of total, electronically gated CD4⁺CD8⁺ (*DP*), CD4⁺CD8⁻ (*4SP*), and CD4⁻CD8⁺ (*8SP*) thymocytes from cultures with immobilized anti-TCR mAb (*broken lines*), immobilized anti-TCR mAb with 10 nM FK506 (*solid lines*), and medium alone (*dotted lines*) are demonstrated with background stainings (*shaded areas*). The percentages of cells present in each area are shown. The mean channel numbers of CD4 and CD8 fluorescence intensity of DP thymocytes were 283/2,811 in the control, 167/2,086 in the anti-TCR alone, and 197/2,342 in the anti-TCR and FK506 treated group. (*Arrow*) CD4^low^CD8^low^ DP thymocytes generated in the culture with anti-TCR. (*B*) Purified B6 DP thymocytes were stimulated in vitro with immobilized anti-TCR mAb in the presence or absence of 10 nM of FK506 for 0.5 or 1 h at 37°C. Total RNA was prepared from the stimulated DP thymocytes and subjected to Northern blot analysis with CD69 and β-actin probes. Arbitrary units are listed under each group as a ratio normalized by the corresponding signal of β-actin.

the shutdown of RAG-1 transcription in DP thymocytes treated with anti-TCR. Purified DP thymocytes were treated with immobilized anti-TCR mAb for 4, 8, and 16 h in the presence or absence of FK506. Northern blot analysis was done with a probe specific for RAG-1. As can be seen in Fig.

5, the decrease of RAG-1 transcription after TCR stimulation was significantly inhibited with FK506. Thus, this event in the developmental processes occurring during positive selection of DP thymocytes appears also to be partially dependent on calcineurin activation.

Effect of FK506 on the Rapid Elevation of [Ca²⁺]ᵢ Induced by Anti-TCR mAb in DP Thymocytes. The rapid elevation of [Ca²⁺]ᵢ induced by anti-TCR mAb, which is an upstream signaling event and is required for calcineurin activation, was not blocked by CsA or FK506 in mature T cells (reviewed in 26). However, the effect of FK506 on the elevation of [Ca²⁺]ᵢ in immature DP thymocytes has not been previously addressed. Thus, we wished to determine the effect of FK506 on the elevation of [Ca²⁺]ᵢ in immature DP thymocytes. Most freshly prepared DP thymocytes express low levels of TCR, and they are poorly functional in response to stimulation with anti-TCR mAb as assessed by calcium mobilization. However, after single cell suspensions of DP thymocytes are cultured in vitro at 37°C for a few hours, they will express increased numbers of TCR and they become competent to mobilize calcium in a manner similar to that of mature T cells (39). Using competent DP thymocytes prepared by this manipulation, we tested the effect of FK506 on the TCR-mediated elevation of [Ca²⁺]ᵢ.

B6 DP thymocytes were separated by panning and precultured in single cell suspensions at 37°C for 2 h. FK506 (10 nM) was added at the beginning of the preculture period when it was to be included in the culture. The precultured DP thymocytes were then loaded with Fura-2, and placed in the reaction chamber of the IMRAS unit. 5 min after overlaying an excess amount of unloaded FcR⁺ LK35.2 cells over the Fura-2–loaded responder thymocytes, [Ca²⁺]ᵢ measurements were collected. At the 1-min time point, anti-CD3 mAb (145-2C11, 50 μg/ml) was added to the cell mixture to activate the thymocytes. FK506 (10 nM) was included in the reaction mixture as well, where appropriate. A representative result of [Ca²⁺]ᵢ in responder DP thymocytes before and 2 min after adding anti-CD3 mAb is shown in Fig. 6. About one half of DP thymocytes expressed high levels of [Ca²⁺]ᵢ in cultures not treated with FK506. This response was not altered by the addition of FK506. We did not observe any significant difference in either the frequency of responding cells or the magnitude of [Ca²⁺]ᵢ in the responding cells in five independent experiments (data not shown). Thus the rapid elevation of [Ca²⁺]ᵢ in DP thymocytes, which occurs as a result of activation by TCR ligation, did not appear to be sensitive to FK506. Similarly, CsA had no effect on the Ca response (data not shown).

Effect of FK506 on T Cell Development in an H-YTg Model. Another system that allows for the evaluation of the effect of FK506 on selection events in the thymus involves the use of transgenic mice that express TCR derived from a H-Y-specific H-2D^b^-restricted CTL clone whose ligand is the male antigen H-Y in the context of H-2D^b^ (5). In the H-2^b^ female H-YTg mouse, thymocytes expressing the transgenic TCR are subject to positive selection in the thymus, whereas the transgene-expressing thymocytes are subject to
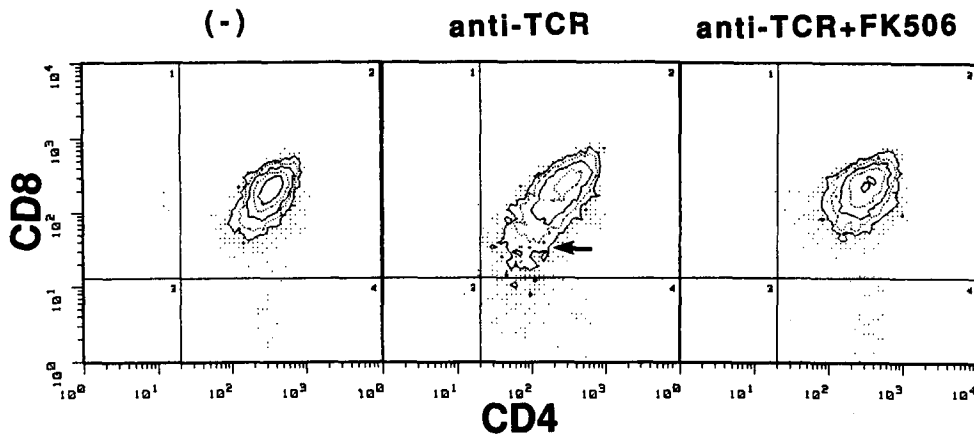
(-)   **anti-TCR**   **anti-TCR+FK506**

CD8

CD4

FK506

0h   4h   8h   16h
        - +   - +   - +

RAG-1

β-Actin

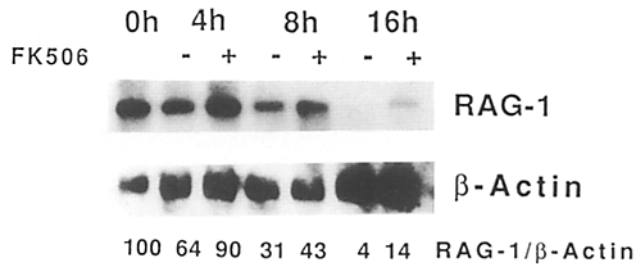100 64 90 31 43   4 14   RAG-1/β-Actin

Figure 5. Effect of FK506 on the shutdown of RAG-1 mRNA in DP thymocytes after stimulation through TCR. Purified B6 DP thymocytes were stimulated in vitro with immobilized anti-TCR mAb in the presence or absence of 10 nM of FK506 for 4, 8, or 16 h at 37°C. Total RNA was prepared from the stimulated DP thymocytes and subjected to Northern blot analysis with RAG-1 and β-actin probes. Arbitrary units are listed under each group as a ratio normalized by the corresponding signal of β-actin.

negative selection in the male thymus because of the expression of both D^b and H-Y antigens. In the H-2^d mouse that bears the TCR transgene, no selection takes place in the thymus because the appropriate restricting element D^b is absent (5).

We investigated the effect of FK506 and CsA on thymocyte development in H-2^b female, H-2^b male, and H-2^d female Tg mice. Tg mice were treated daily with 10 μg of FK506 or 30 μg of CsA for 2 wk from birth. CD4/CD8 profiles of electronically live-gated Tg TCRα^+ (T3.70^+) thymocytes were obtained by three-color FCM analysis with anti-CD4-PE, anti-CD8-APC, and anti-T3.70-FITC. As shown in Fig. 7, top, the generation of Tg CD8 SP thymocytes in H-2^b female mice was severely inhibited by the treatment of FK506. Yields of thymocytes between PBS- and FK506-treated groups were similar. Therefore, similar to the result obtained in normal B6 mice treated with FK506, positive selection of the H-YTg
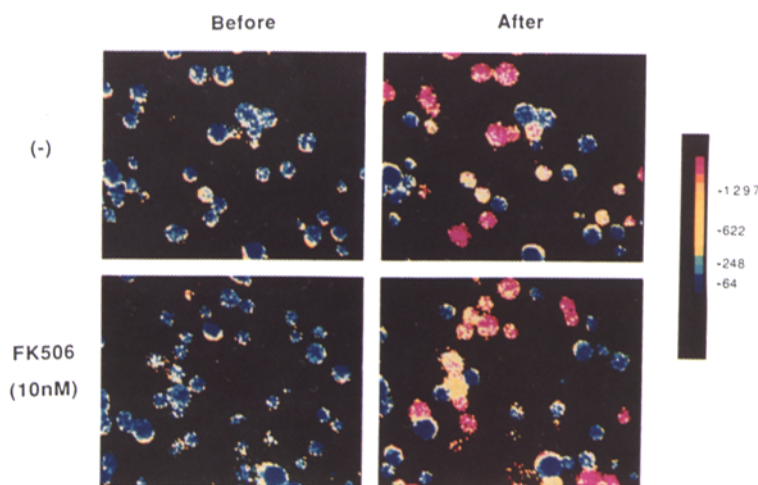

Before   After

(-)

FK506
(10nM)

-1297
-622
-248
-64

Figure 6. Effect of FK506 on the rapid elevation of [Ca^2+]_i induced by anti-CD3 mAb in DP thymocytes. Purified B6 DP thymocytes were cultured in single suspensions in vitro at 37°C for 2 h. The cultured cells were loaded with Fura-2, placed on a reaction chamber, and then overlaid with an excess number of FcR^+ LK35.2 cells. Stimulation was done by adding anti-CD3 mAb (145-2C11) to the reaction mixture. [Ca^2+]_i of responder DP thymocytes was determined by fluorescence microscopy and digital image analysis. [Ca^2+]_i of DP thymocytes before (*Before*) and 2 min after the stimulation (*After*) are demonstrated. The hue of the cells in the picture represents [Ca^2+]_i, whose scale (nanomolar) is shown on the right.
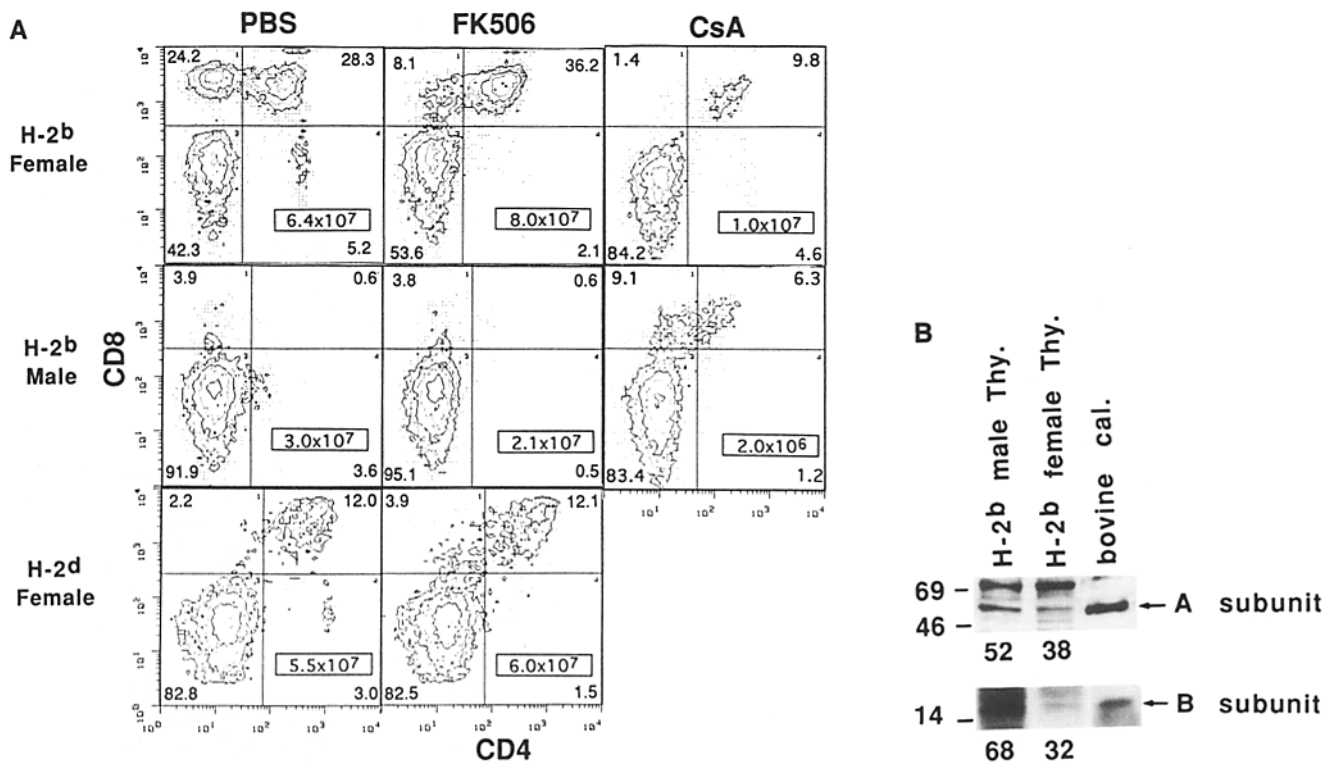
Figure 7. Effect of FK506 and CsA on T cell development in H-YTg thymus and the amount of calcineurin in H-YTg thymocytes. H-2b female, H-2b male, and H-2d female H-YTg neonates were treated daily with 10 μg of FK506 or 30 μg of CsA for 2 wk. Thymocytes were stained with anti-CD4-PE, anti-CD8-APC, and anti-T3.70-FITC. CD4/CD8 profiles of electronically gated Tg TCRα+ (T3.70+) thymocytes are depicted. The yield of thymocytes in each mouse is shown as boxed numbers. The percentage of cells present in each area are also shown. (B) NP-40 lysate of thymocytes (10^7 cells/lane) from H-2b male and female H-YTg mice, and purified bovine calcineurin (0.1 μg) were subjected to immunoblotting with anticalcineurin antibodies. (Arrows) A and B subunits. (Left) Position of molecular weight markers (kilodaltons). Arbitrary densitometric units are listed under each band.

Table 1. *Effect of FK506 on the Intrathymic Deletion of Vβ6 and Vβ11 Cells in CBA/J Mice*

Mouse strain	Dose of FK506	Percentage of CD4 SP thymocytes	Percentage of TCRVβ positive cells in CD4 SP thymocytes		
			Vβ6	Vβ11	Vβ8.2
	μg				
Exp. 1					
B6	0	6.3	6.4	4.2	9.7
CBA/J	0	4.7 ± 0.9	0.25 ± 0.4	0.58 ± 0.4	11.8 ± 1.4
CBA/J	1	3.6 ± 1.4	0.23 ± 0.3	0.13 ± 0.2	12.7 ± 1.2
CBA/J	3	1.9 ± 0.3	0.06 ± 0.1	0.27 ± 0.3	13.6 ± 2.5
Expt. 2					
B6	0	5.7	7.7	3.6	6.8
CBA/J	0	5.7 ± 2.7	0.13 ± 0.1	0.74 ± 0.1	12.4 ± 2.2
CBA/J	1	2.9 ± 1.2	0.21 ± 0.2	0.23 ± 0.2	15.9 ± 0.5
CBA/J	3	2.0 ± 0.1	0.22 ± 0.4	0.21 ± 0.3	12.4 ± 1.6

A B6 neonatal mouse and CBA/J neonates (four neonates per group) were treated with indicated doses of FK506 for 2 wk. Thymocytes were stained with anti-CD4-PE, anti-CD8-APC, and one of the culture supernatants of mAb specific for TCRVβ6 (44-22-1), TCRVβ11 (RR3-15), and TCRVβ8.2 (F23.2). Goat anti-rat Ig-FITC was used for anti-TCRVβ6 and anti-TCRVβ11 stainings. Goat anti–mouse Ig-FITC was used for anti-TCRVβ8.2 staining. Percentages of CD4 SP thymocytes were calculated from 20,000 viable cells. Percentages of Vβ6, Vβ11, and Vβ8.2 cells in CD4 SP thymocytes are demonstrated with standard deviations.

T cells appeared to be sensitive to FK506-dependent calcineurin activation. More interestingly, treatment with FK506 did not affect CD4/CD8 profiles of thymocytes of H-2^b male Tg mice (Fig. 7, *middle*). Since the development of DP thymocytes in H-2^b or H-2^d female Tg mice (Fig. 7, *bottom*) was not inhibited by FK506 treatment, the most straightforward explanation of the result in male mice was that negative selection of the H-YTg TCR$^+$ cells was not inhibited by FK506 treatment. A small amount of CD4 SP thymocytes present in H-2^b female and H-2^d female mice was most probably Tg TCRα$^-$ cells which could not be excluded by gating with T3.70 staining (data not shown). The development of this CD4 SP population was inhibited in mice treated with FK506.

In contrast to the yields of thymocytes in the FK506-treated group, those from CsA-treated animals were about 1/6 to 1/10 in number. Although certain proportions of CD8 SP and DP thymocytes were observed in CsA-treated Tg male mice, the absolute number of the cells present in the thymus was not significantly higher than that in the PBS-treated group. Taken together, these results suggested that the effect of CsA was on the generation or survival of DN thymocytes rather than a rescue of Tg TCR$^+$ thymocytes from negative selection by H-Y antigen.

Expression of Calcineurin in Thymocytes from H-2^b Male and Female H-YTg Mice. Next, the amount of calcineurin present in thymocytes from H-2^b male and female H-YTg mice was determined by immunoblotting. NP-40 lysates of thymocytes from H-2^b male and female H-YTg mice and purified bovine calcineurin (as a positive control) were subjected to SDS-PAGE and, after electrotransfer, the blots were probed with anticalcineurin antibodies. The amounts of A and B subunits present in the lysates of thymocytes were determined by densitometric analysis of autoradiographs and are demonstrated in Fig. 7 *B*. Thymocytes from male mice contained higher amounts of both calcineurin A and B subunits.

Effect of FK506 on the Intrathymic Deletion of Vβ6 and Vβ11 Cells in CBA/J Mice. Negative selection of self-reactive T cells in the thymus is also a result of TCR ligation of self-peptides presented on MHC molecules on thymic stromal cells. We continued to examine the requirement of calcineurin activation in negative selection using other experimental systems. One system involves the intrathymic clonal deletion of Vβ6 cells and Vβ11 cells in CBA/J mice (Mls-1^a, I-E$^+$) (4). Since FK506 treatment blocks the generation of mature SP thymocytes, we treated CBA/J neonates with suboptimal doses of FK506 in order to assess the effect of FK506 on the selection of specific Vβ-bearing thymocytes. Three-color FCM analysis was done with anti-CD4, anti-CD8, and mAb specific for TCRVβ6, Vβ11, or Vβ8.2. Percentages of CD4 SP cells and percentages of Vβ6, Vβ11, and Vβ8.2 cells in the CD4 SP cells were calculated. The generation of CD4 SP thymocytes was inhibited partially but, nevertheless, in a dose-dependent fashion by FK506 (Table 1). In contrast to the control B6 mice, only a small number of Vβ6 and Vβ11 cells in CD4 SP thymocytes was observed among CBA/J thymocytes. No detectable rescue of CD4 SP thymocytes expressing

Vβ6 or Vβ11 TCR was observed under these conditions. Percentages of Vβ8.2 cells in the FK506-treated groups did not differ from those of untreated mice. Thus, intrathymic clonal deletion of Vβ6 and Vβ11 cells in CBA/J mice appeared to be resistant to FK506 treatment.

Effect of FK506 on Anti-TCR mAb- and SEB-induced DNA Fragmentation in DP Thymocytes. Another system to evaluate the effect of FK506 on negative selection of thymocytes is through the analysis of in vitro experimental models of negative selection (49). Consequently, we examined the effect of FK506 on DNA fragmentation in DP thymocytes induced by stimulation through the TCR in vitro. Purified DP thymocytes from normal B6 mice were first precultured for 2 h and then stimulated for 4 h by FcR$^+$ LK35.2 cells preincubated with anti-TCRαβ mAb (H57-597) or anti-CD3 mAb (145-2C11) as described (49). Another group of precultured DP thymocytes was treated with 1 μM hydrocortisone. When FK506 was included, it was added at the beginning of the preculture at a dose of 10 nM. Genomic DNA from the stimulated DP thymocytes was extracted and analyzed on 0.8% agarose gels. As shown in Fig. 8 *A*, DNA fragmentation was induced in DP thymocytes stimulated with anti-TCRαβ mAb or anti-CD3 mAb in the presence of FcR$^+$ cell line LK35.2. The DNA fragmentation induced by these TCR stimulations was not affected by FK506. In addition, steroid-induced DNA fragmentation also appeared to be resistant to FK506 treatment.

In a second model, DP thymocytes were stimulated with the superantigen SEB, and the effect of FK506 was examined. To increase the number of SEB-reactive cells, DP thymocytes were purified from TCRVβ8.2 Tg mice. These cells were subsequently stimulated with SEB and I-E^k transfectant DCEK cells as described (49). As shown in Fig. 8 *B*, DNA fragmentation was observed in the cells stimulated with SEB plus DCEK, and this was not inhibited in the presence of FK506. In another experiment, the effects of CsA and EGTA were examined (Fig. 8 *C*). As expected, no effect of CsA on the DNA fragmentation in DP thymocytes induced by anti-TCR mAb was detected. In the same stimulation cultures, partial inhibition of DNA fragmentation in DP thymocytes was observed with EGTA. Since EGTA abrogates extracellular calcium ions, this result suggests that calcium influx may play an important role in DNA fragmentation in DP thymocytes. As a positive control of treatment of FK506 and CsA, we performed similar experiments using T cell hybridoma 68-41. In contrast to the results with DP thymocytes, both FK506 and CsA blocked the anti-TCR-induced DNA fragmentation in the T cell hybridoma 68-41 (Fig. 8 *D*). This is consistent with previous reports (54, 55). Taken together, the DNA fragmentation induced in DP thymocytes by anti-TCR mAb, anti-CD3 mAb, SEB, or steroid appears to be independent of calcineurin activation. In addition, it is interesting that the DNA fragmentation that occurs in mature T cells (in this case a T cell hybridoma) as a result of anti-TCR induction is sensitive to FK506 and CsA, and thus appears to be dependent on calcineurin activation. Thus, the results demonstrated in Fig. 8 suggest that the signal trans-
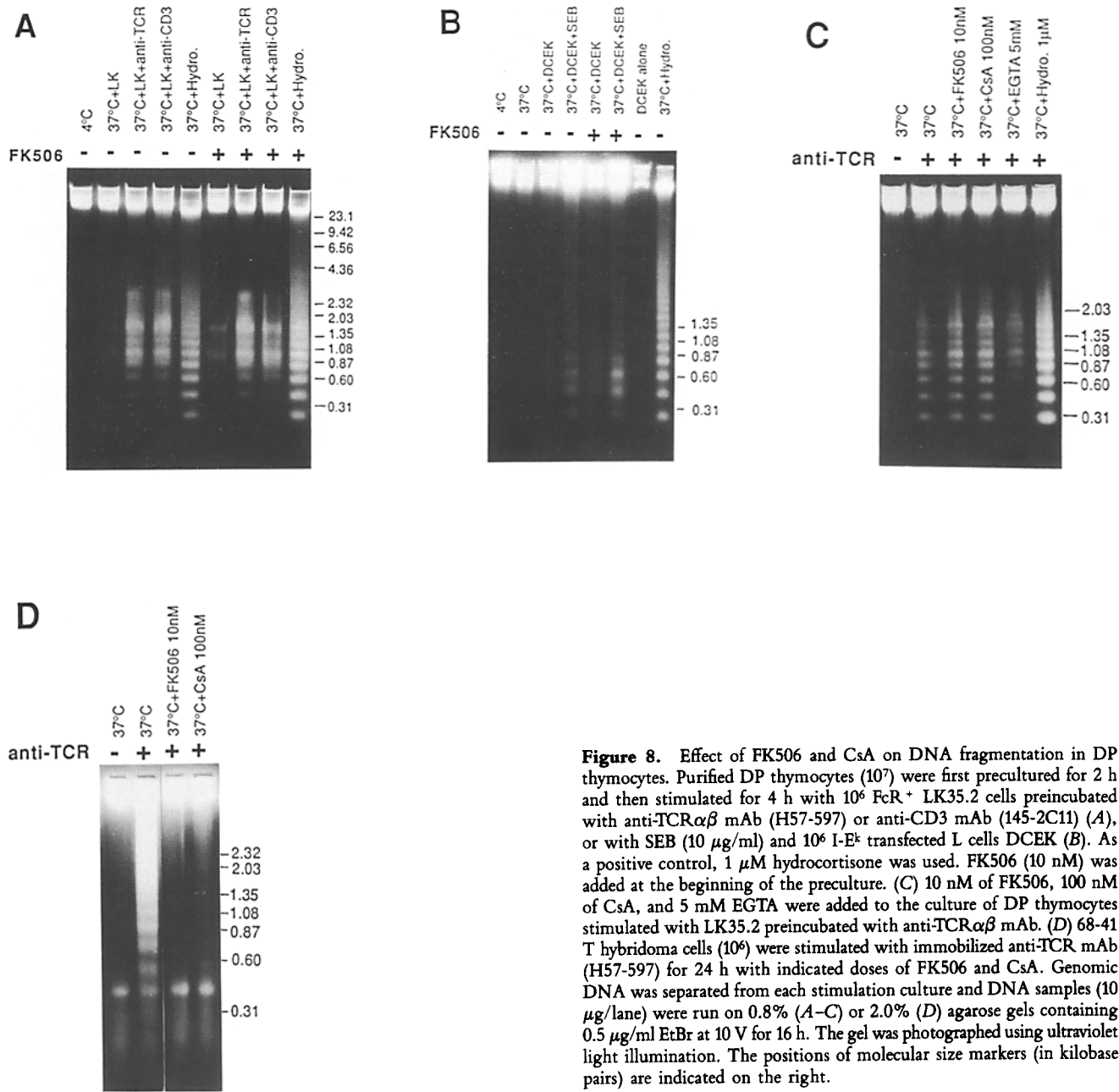
A

FK506 − − − − − + + + +

Lane labels: 4°C | 37°C+LK | 37°C+LK+anti-TCR | 37°C+LK+anti-CD3 | 37°C+Hydro. | 37°C+LK | 37°C+LK+anti-TCR | 37°C+LK+anti-CD3 | 37°C+Hydro.

Markers: 23.1, 9.42, 6.56, 4.36, 2.32, 2.03, 1.35, 1.08, 0.87, 0.60, 0.31

B

FK506 − − − − − + + − −

Lane labels: 4°C | 37°C | 37°C+DCEK | 37°C+DCEK+SEB | 37°C+DCEK | 37°C+DCEK+SEB | DCEK alone | 37°C+Hydro.

Markers: 1.35, 1.08, 0.87, 0.60, 0.31

C

anti-TCR − + + + + +

Lane labels: 37°C | 37°C | 37°C+FK506 10nM | 37°C+CsA 100nM | 37°C+EGTA 5mM | 37°C+Hydro. 1μM

Markers: 2.03, 1.35, 1.08, 0.87, 0.60, 0.31

D

anti-TCR − + + +

Lane labels: 37°C | 37°C | 37°C+FK506 10nM | 37°C+CsA 100nM

Markers: 2.32, 2.03, 1.35, 1.08, 0.87, 0.60, 0.31

Figure 8. Effect of FK506 and CsA on DNA fragmentation in DP thymocytes. Purified DP thymocytes (10^7) were first precultured for 2 h and then stimulated for 4 h with 10^6 FcR$^+$ LK35.2 cells preincubated with anti-TCR$\alpha\beta$ mAb (H57-597) or anti-CD3 mAb (145-2C11) (*A*), or with SEB (10 μg/ml) and 10^6 I-E^k transfected L cells DCEK (*B*). As a positive control, 1 μM hydrocortisone was used. FK506 (10 nM) was added at the beginning of the preculture. (*C*) 10 nM of FK506, 100 nM of CsA, and 5 mM EGTA were added to the culture of DP thymocytes stimulated with LK35.2 preincubated with anti-TCR$\alpha\beta$ mAb. (*D*) 68-41 T hybridoma cells (10^6) were stimulated with immobilized anti-TCR mAb (H57-597) for 24 h with indicated doses of FK506 and CsA. Genomic DNA was separated from each stimulation culture and DNA samples (10 μg/lane) were run on 0.8% (*A–C*) or 2.0% (*D*) agarose gels containing 0.5 μg/ml EtBr at 10 V for 16 h. The gel was photographed using ultraviolet light illumination. The positions of molecular size markers (in kilobase pairs) are indicated on the right.

duction pathways leading to DNA fragmentation in immature DP thymocytes are different from those in mature T cell hybridomas.

To address the effect of these inhibitory drugs on apoptotic cell death of DP thymocytes more quantitatively, we used a thymocyte death assay using EtBr and FCM as described (50, 51). EtBr is rapidly taken up by thymocytes that are destined to die, and binds to nucleic acid. The fluorescence emitted can be measured on a single cell basis by FCM analysis. EtBr$^+$ cells fluoresce with two different intensities (EtBr^{int} and EtBr^{high}), both of which represent dying cells in which DNA fragmentation is present (50, 51). Precultured thymocytes (2 h at 37°C) were stimulated by LK35.2 cells

preincubated with anti-TCR or anti-CD3 in the presence of the indicated drugs for another 4 h. Where indicated, the precultured thymocytes were stimulated with thapsigargin, which causes release of Ca^{2+} from intracellular stores. The stimulated cells were stained first with anti-CD4-PE and anti-CD8-APC, and then stained with 1 μg/ml of EtBr for 30 min. Percentages of EtBr$^+$ cells in electronically gated DP cell populations are shown in Fig. 9. 25–30% of DP thymocytes stimulated with anti-TCR mAbs for 4 h were positive for EtBr. The addition of FK506 or CsA had no effect on these cultures. In contrast, the addition of either EGTA or EDTA inhibited the TCR-induced increase of EtBr$^+$ cells to the background level. This is consistent with the data presented
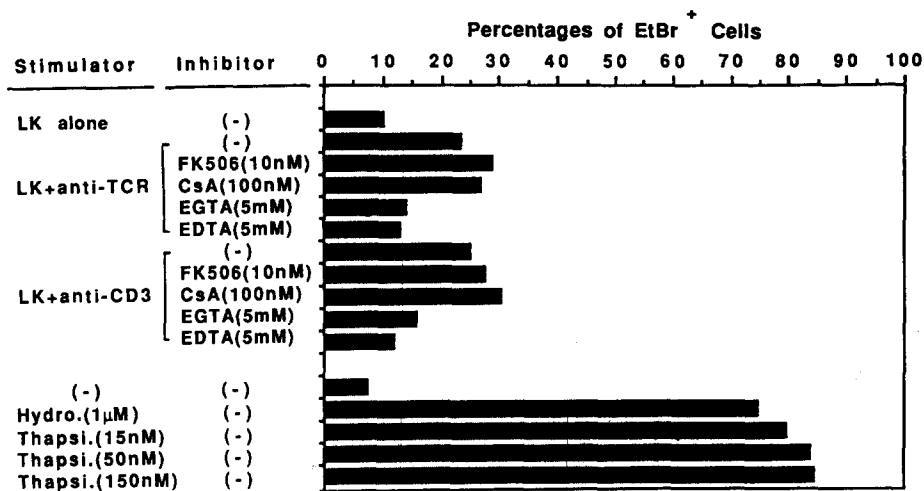
Percentages of EtBr$^+$ Cells

Stimulator	Inhibitor
LK alone	(-)
LK+anti-TCR	(-)
	FK506(10nM)
	CsA(100nM)
	EGTA(5mM)
	EDTA(5mM)
LK+anti-CD3	(-)
	FK506(10nM)
	CsA(100nM)
	EGTA(5mM)
	EDTA(5mM)
(-)	(-)
Hydro.(1μM)	(-)
Thapsi.(15nM)	(-)
Thapsi.(50nM)	(-)
Thapsi.(150nM)	(-)

Figure 9. Cell death of DP thymocytes stimulated with TCR/CD3 complexes assayed by EtBr staining and FCM analysis. Precultured thymocytes (2 × 10^6, 2 h at 37°C) were stimulated by 4 × 10^5 LK35.2 cells preincubated with anti-TCR (H57-597) or anti-CD3 (145-2C11) for 4 h at 37°C. Indicated drugs were added at the beginning of each preculture. Where indicated, the precultured thymocytes were stimulated with graded doses of thapsigargin (*Thapsi.*), which causes the release of Ca^{2+} from intracellular stores. The stimulated cells were harvested, stained with anti-CD4-PE and anti-CD8-APC, and then exposed to 1 μg/ml of EtBr for 30 min at 4°C. Percentages of EtBr$^+$ cells in electronically gated DP cell population are shown.

in Fig. 8 C. In addition, treatment with thapsigargin induced apoptotic cell death quite strongly. Therefore, calcium-dependent intracellular enzymes other than calcineurin may be involved in the signal pathway that leads to events resulting in apoptotic cell death accompanied by DNA fragmentation.

Discussion

Selection of the TCR repertoire has been quite paradoxical because of its contradictory nature. T cells that recognize self-MHC molecules are deleted during development in the thymus in a process referred to as negative selection. However, recognition of self-MHC molecules is also required for positive selection. One explanation of this paradox is that the affinity of the TCR for peptide presented on MHC molecules determines the fate of T cells, i.e., positive or negative selection. Weak interactions between TCR and peptide-MHC would be sufficient to mediate positive selection, whereas, higher affinity interactions would lead to negative selection (56). A second model proposes that both positive and negative selection are induced by high affinity interactions but that there are qualitative differences between the peptide/MHC complexes·that mediate positive and negative selection (57). There are many experiments that support the notion that the thymic epithelial cells are responsible for positive selection, whereas the bone marrow–derived cells mediate negative selection (reviewed in 56, 57). If thymic epithelial cells express a distinct set of peptide/MHC complexes, positive selection could be mediated by qualitatively different peptide/MHC complexes. This view would provide a resolution of the paradox in which both positive and negative selection result from the recognition of self-MHC molecules in the thymus. Recently, experiments using antigenic peptides in fetal thymus organ cultures of TCR transgenic and MHC-deficient mice have been carried out by several groups (58, 59), and the results supported the former so called "affinity model." It was demonstrated clearly that low concentrations of peptide induced positive selection, whereas high concentrations of the same peptide induced negative selection. Thus,

thymocytes responded with qualitatively different outputs to different intensity signals. It is possible that thymocytes can be induced to mature (positive selection) by a weak interaction whereas strong interactions may activate apoptotic cell death (negative selection). Therefore, it is important to investigate the nature of the intracellular signals generated during thymic selection processes, and the differences that determine the positive and negative selection.

In this report, we examined TCR-mediated early signaling events in CD4$^+$CD8$^+$ DP thymocytes during positive and negative selection by using an inhibitor of calcineurin activation, FK506, to specifically address the role of calcineurin activation in the selection process. Two major findings were demonstrated. First, calcineurin activation induced by stimulation through the TCR in DP thymocytes is an essential event for the maturation of SP cells, and is required for the first step (or the very early intracellular signaling events) of the positive selection processes. During the first step of positive selection, induction of CD69 molecule, downregulation of CD4 and CD8 expression, and shutdown of RAG transcription take place. These changes were significantly inhibited by the treatment with FK506. Second, different signaling pathways are operative between thymic positive and negative selection in terms of the dependency on calcineurin activation. In contrast to the effect on positive selection, negative selection in the thymus and in vitro–induced DNA fragmentation in DP thymocytes were not affected by the treatment with FK506.

As we reported recently (11), the thymocyte subpopulation undergoing positive selection can be identified as CD69-positive DP thymocytes with increased expression of TCR and decreased expression of both CD4 and CD8 molecules. DP thymocytes with this phenotype were induced by stimulation with anti-TCR mAb in vitro (11). RAG-1 and RAG-2 transcription was detected in immature TCR^{low} DP thymocytes but not in TCR^{int} DP thymocytes or mature SP thymocytes, and the transcription of these genes were shutdown upon stimulation with anti-TCR mAb in vitro (20, 21). Therefore, the induction of CD69, downregulation of

CD4 and CD8 expression, and the decrease of the RAG expression in DP thymocytes are thought to be consequences of TCR-mediated signals. As we demonstrated, the CD69$^+$ DP thymocyte subpopulation was not detected in the thymus of mice treated with FK506 (Fig. 2). Transcription and cell surface expression of CD69 were induced in DP thymocytes within a few hours after anti-TCR stimulation in vitro, and these events were significantly inhibited by FK506 (Fig. 3). The decrease in cell surface expression of both CD4 and CD8 that is induced by the anti-TCR stimulation in vitro was also blocked by FK506 (Fig. 4). Furthermore, the shutdown of RAG-1 transcription in the stimulated DP thymocytes was significantly inhibited by FK506 (Fig. 5). Taken together, these results suggest that FK506 blocks the generation of mature T cells by inhibiting the calcineurin activation pathway in DP thymocytes activated through the TCR, which is required for the first step of thymic positive selection. Partial inhibitions observed in in vitro experiments (Figs. 3 and 5) might reflect redundancy of signal transduction pathways.

Both thymic positive and negative selection are thought to be consequences of intracellular signaling events initiated by TCR engagement of ligands on thymic stromal cells. We examined the effect of FK506 on thymic negative selection using three different experimental systems. First, we tested the effect of FK506 on negative selection in a H-YTg model. In the H-YTg male mice, male antigen–specific Tg TCR-positive T cells are deleted in the thymus, and only CD4$^-$ CD8$^-$ thymocytes are detected (5). If this negative selection is blocked by FK506, the appearance of DP thymocytes would be the anticipated result because the generation of these DP thymocytes is not affected by the treatment with FK506. We did not detect any rescue of DP thymocytes in the FK506 treated H-YTg neonates (Fig. 5). These results suggest that calcineurin activation is not critical for negative selection. In terms of the interpretation of the results with CsA (Fig. 7 A, right), we drew different conclusions from those of Urdahl et al. (60). Both they and we observed a certain percentage of DP thymocytes in H-YTg male mice treated with CsA. Since the absolute numbers of the DP thymocytes present in the CsA-treated neonates were not significantly higher than those in the untreated group, we did not interpret the result to indicate any rescue or delay of negative selection by the treatment with CsA. Urdahl et al. (60) concluded that CsA delays negative selection of H-YTg thymocytes in H-2^b male mice. The reason for the discrepancy is not clear, although it could be due to the use of different experimental systems. In this study, neonatal H-YTg mice were used whereas Urdahl et al. (60) used adult H-YTg mice. In any case, it may be more appropriate to use FK506 rather than CsA for investigating the role of calcineurin activation in thymic selection process. It is clear that the FK506-sensitive calcineurin is not involved in the negative selection of H-YTg thymocytes. The differences in effect of CsA and FK506 on DN thymocytes are discussed below.

The second system in which the effect of FK506 on negative selection was tested involved the clonal deletion of Vβ6-TCR, Vβ11-TCR positive cells in CBA/J (Mls-1^a, I-E$^+$)

mice. In CBA/J (Mls-1^a, I-E$^+$) mice, Vβ6-TCR, Vβ11-TCR positive cells are deleted within the DP thymocyte population because of the ligation of the TCR with the endogenous superantigen, and no Vβ6$^+$ or Vβ11$^+$ SP thymocytes is detected (4). We did not detect rescue of Vβ6$^+$ or Vβ11$^+$ cells in CD4 SP thymocyte subpopulation in FK506-treated CBA/J mice (Table 1). Jenkins et al. (24) reported the presence of undeleted mature SP thymocytes, i.e., Vβ17a$^+$ cells, in CsA-treated C57BR (I-E$^+$) syngeneic mouse bone marrow chimeras. Gao et al. (23) also reported incomplete deletion of peripheral TCR Vβ11$^+$ T cells in I-E–bearing CsA-treated syngeneic mouse bone marrow chimeras. In contrast, Prud'homme et al. (61) reported that there were no significant numbers of T cells bearing forbidden TCRs in the spleen of either CsA-treated CBA/J, C57BR, or DBA/2 syngeneic bone marrow chimeras. Bryston et al. (62) also reported that deletion of various Vβs in DBA/2 mice was not blocked by CsA. The reasons for the discrepancies of results among the different experimental systems are not clear. One explanation is that a small number of undeleted TCR Vβ-bearing T cells detected in the periphery by Gao et al. (23) might be a consequence of the blocking of activation-induced cell death of mature T cells (54, 55). In fact, DNA fragmentation in T cell hybridoma 68-41 was sensitive to either FK506 or CsA (Fig. 8).

Thymocytes are thought to be deleted by apoptosis, a process characterized by DNA fragmentation (63). Consequently, we examined the FK506 effect on DNA fragmentation in DP thymocytes by TCR stimulation in vitro. The results presented in Fig. 8 clearly demonstrated that DNA fragmentation in DP thymocytes induced by stimulation with anti-TCR mAb, anti-CD3 mAb, superantigen SEB, or steroid was not affected by treatment with FK506. Therefore, from the results obtained in these three different systems, we conclude that FK506-sensitive calcineurin activation was not required for negative selection of thymocytes.

Since fragmented DNA is produced by the activation of calcium-dependent endonucleases (reviewed in 64), the increased level of [Ca^{2+}]$_i$ is thought to be important for apoptotic cell death. After stimulation through the TCR, rapid increase in [Ca^{2+}]$_i$ was detected in DP thymocytes if they were desensitized by a 37°C preculture (39 and Fig. 6). In addition, the frequencies of cells expressing high [Ca^{2+}]$_i$ are increased in the thymus of TCR Tg mice that bear a negatively selecting environment (46). Abrogation of extracellular Ca^{2+} by addition of EGTA inhibited the DNA fragmentation (Fig. 8 C) and TCR-induced cell death of DP thymocytes (Fig. 9). Thapsigargin, which causes release of the intracellular calcium stores, induced cell death in DP thymocytes (Fig. 9). The rapid increase in [Ca^{2+}]$_i$ after stimulation through the TCR on DP thymocytes was not affected by treatment with FK506 (Fig. 6). Taken together, signaling pathways leading to DNA fragmentation in DP thymocytes appeared to be dependent on an increase in [Ca^{2+}]$_i$ but not on calcineurin activation.

In experiments examining in vitro DNA fragmentation, another interesting result was observed in that FK506 and

CsA showed different effects on DNA fragmentation induced in DP thymocytes as compared with that induced in T hybridoma cells. DNA fragmentation induced in T hybridoma cells by stimulation through the TCR was shown to be blocked by CsA (54, 55). This observation was confirmed in our study also. However, since DNA fragmentation in DP thymocytes induced by stimulation through the TCR was not inhibited, calcineurin activation events may differentiate signaling pathways in the DNA fragmentation process in T cells as well.

If thymic negative selection is mediated by so-called high affinity interactions and relatively weaker stimulations result in positive selection, it is also possible that the signals generated during negative selection processes are strong enough to overcome the blocking effect of calcineurin activation by FK506. In fact, the amount of calcineurin present in H-YTg male thymocytes appeared to be higher than that in H-YTg female thymocytes. However, this possibility is unlikely because the dose of FK506 used in this study is at least 100 times higher than that in which a complete inhibition of proliferation of mature T cells is observed.

The CD4^{low}CD8^{low} phenotype and expression of CD69 in DP thymocytes are characteristic of the cells undergoing negative as well as positive selection (11). Although FK506 inhibited the generation of these cells both in vivo and in vitro (Figs. 2–4), negative selection appeared not to be inhibited. Therefore, the downmodulation of both CD4 and CD8 molecules and the induction of CD69 expression might not be essential events for proceeding thymic negative selection.

The final point which should be discussed is the difference in effect of CsA and FK506 on T cell development in the thymus. CsA and FK506 are quite different in structure, and their binding proteins, cyclophylin and FKBP, respectively, are also very different in character. However, both drug–protein complexes were found to block calcineurin activation (reviewed in 26), a surprise for researchers in the field. Here, we demonstrated some differences in the effects of FK506 and CsA on T cell development in the thymus. As shown in Fig. 1, yields of thymocytes were decreased when neonatal mice were treated with CsA but not with FK506. CsA blocked the generation of DP thymocytes. This was not observed with FK506. In addition, CsA showed an inhibitory effect on the generation or survival of DN thymocytes, that was observed in both normal B6 (Fig. 1) and H-YTg neonates (Fig. 7 A). Thus, CsA-sensitive signaling pathways appeared to be required for T cell maturation to DP thymocytes, and important for the generation of DN thymocytes. FK506 did not show these effects, and therefore CsA might have other unknown consequences in immature thymocytes.

In summary, the role of calcineurin activation during thymic selection events was investigated through the use of the inhibitor, FK506. The results obtained demonstrated that TCR-mediated calcineurin activation in DP thymocytes is an essential event in the first step of the TCR-specific positive selection processes, and that calcineurin activation is not required for negative selection of immature DP thymocytes.

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