

Selective Apoptosis of CD4⁺CD8⁺ Thymocytes by the Anti-Fas Antibody

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Summary

Fas is a cell surface protein that mediates apoptosis. A mouse mutant, *lpr* (lymphoproliferation), has a mutation in the Fas gene. In this report, we studied the expression and function of Fas in various subpopulations of mouse thymocytes. Abundant expression of Fas was detected on CD4⁺CD8⁺ double positive as well as CD4⁺ or CD8⁺ single positive thymocytes in wild-type mice. Little or low levels of Fas were expressed in CD4⁻CD8⁻ double negative thymocytes except for the CD4⁻CD8⁻CD3⁺ phenotype, which expresses Fas as abundantly as double positive or single positive subsets. On the other hand, no Fas expression was detected in any population of thymocytes from *lpr* mice. When the wild-type thymocytes were treated with the agonistic anti-Fas antibody, double positive cells from the wild-type mice were selectively killed by apoptosis, whereas, the single positive cells were resistant to its cytolytic activity despite their abundant expression of Fas. Unlike the apoptosis of thymocytes induced by glucocorticoid or T cell activator, the Fas-induced apoptosis of thymocytes was enhanced by metabolic inhibitors such as cycloheximide. Furthermore, intraperitoneal administration of the anti-Fas antibody into mice caused rapid apoptosis of thymocytes *in vivo*.

T lymphocytes respond to a wide variety of foreign antigens, whereas they do not respond to self components. This repertoire of T cells are generated during their development in the thymus (1). The interaction of immature CD3⁺/_{low} CD4⁺CD8⁺ thymocytes with epithelial stroma cells in the thymus induces positive selection of T cells, in which T cells recognizing the self-MHC as recognition elements are positively selected and leave the thymus for the periphery (2). The T cells which do not undergo positive selection die by apoptosis. The immature thymocytes are also subject to negative selection in which the high avidity interaction of thymocytes with the thymic stromal cells eliminates the T cells that are potentially autoreactive (3). This process is called clonal deletion and proceeds by apoptosis. In addition to the selection mechanism in the thymus, mature T cells recognizing the self-components are extrathymically eliminated by apoptosis (4).

The Fas antigen (Fas) is a cell-surface protein with molecular mass of 45 kD, and it belongs to the TNF/nerve growth factor (NGF)¹ receptor family (5–7). Since agonistic anti-Fas antibodies or Fas ligand can induce apoptosis under most circumstances (8–12), the Fas ligand and Fas can be regarded as a death factor and its receptor (7). Fas is expressed in var-

ious tissues such as the thymus, liver, heart and ovary (5), whereas the Fas ligand is abundant in activated splenocytes and the testis (9). Chromosomal mapping and molecular analyses of the Fas and Fas ligand genes have indicated that mouse mutants, lymphoproliferation (*lpr*) and generalized lymphoproliferative disease (*gld*) carry mutations in the Fas and Fas ligand, respectively (13–15). Mice homozygous for these mutations have similar phenotypes (16). They accumulate a large number of abnormal T lymphocytes (CD4⁻CD8⁻) in the lymph nodes and spleen, and suffer from autoimmune disease by producing autoantibody. Since *lpr* and *gld* are loss-of-function mutations of Fas and Fas ligand, these phenotypes indicate that the Fas and Fas ligand system is involved in the deletion process of autoreactive T cells.

To examine the expression and function of Fas in mice, we established an agonistic monoclonal antibody against mouse Fas that has cytotoxic activity *in vitro* and *in vivo* (8). In this report, we examined the Fas expression of mouse thymocytes and their sensitivity to the cytotoxic activity of the antibody. Although most thymocytes except for the CD3⁻CD4⁻CD8⁻ subpopulation uniformly express Fas, only CD4⁺CD8⁺ double positive (DP) thymocytes were susceptible to the cytotoxic activity of the anti-Fas antibody.

Materials and Methods

Antibodies. The hamster hybridoma Jo2 producing anti-mouse Fas antibody (8) was cultured in AIM-V medium, and the mono-

¹ Abbreviations used in this paper: DN, double negative cells; DP, double positive cells; *gld*, generalized lymphoproliferative disease; *lpr*, lymphoproliferation; NGF, nerve growth factor; SP, single positive cells.

clonal antibody was purified from the cultured supernatant by affinity chromatography on protein A-Sepharose as described (8). In some cases, the antibody was used after biotinylation (8). Rat anti-CD5 monoclonal antibody (clone, 53-7.3) was purchased from PharMingen (San Diego, CA). PE-conjugated goat anti-hamster IgG and streptavidin-TRICOLOR were purchased from Caltag (San Francisco, CA). Biotinylated or PE-conjugated rat anti-L3T4 (CD4) monoclonal antibody (clone, Gk 1.5) were from Becton Dickinson Immunocytometry Systems (San Jose, CA). FITC-conjugated or PE-conjugated rat anti-Lyt-2 (CD8) monoclonal antibody (clone, 53-6.7) were from Becton Dickinson or PharMingen, respectively. FITC-conjugated rat anti-CD3 monoclonal antibody (145-2C11) was purchased from Boehringer Mannheim Corp. (Indianapolis, IN).

Mice and Preparation of Thymocytes. BALB/c-+/+, MRL/MpJ-

+/+, and MRL/MpJ-*lpr/lpr* mice were purchased from Shizuoka Laboratory Animal Cooperation (Hamamatsu, Japan). CBA/K1-+/+ and CBA/K1-*lpr^g/lpr^g* mice were obtained from Dr. A. Matsuzawa (Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan). All mice used were 3–7 wk old. To prepare thymocytes, thymuses surgically excised from mice were pressed between glass slides, and the cells were suspended in RPMI 1640 medium containing 10% FCS. The cell suspension was then filtered through nylon mesh to remove debris.

Flow Cytometry. Single thymocytes were suspended in staining solution (PBS containing 2% FCS and 0.02% sodium azide), and washed twice with the same solution. Aliquots of $\sim 10^6$ cells were stained by incubation with optimal concentrations of the antibodies described above for 30 min at 4°C in 100 μ l of staining solution. After washing twice with staining solution, the cells were incubated

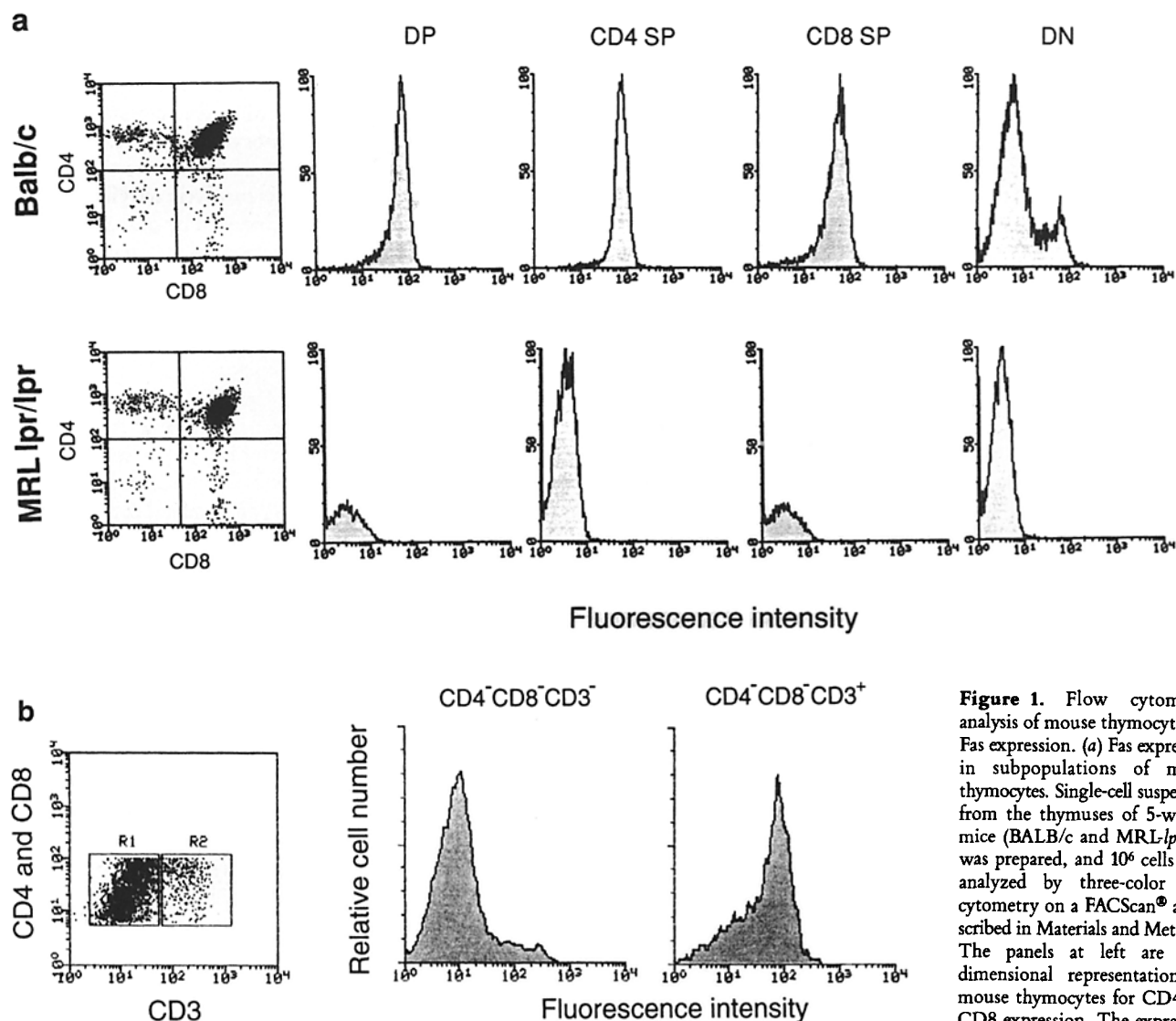


Figure 1. Flow cytometric analysis of mouse thymocytes for Fas expression. (a) Fas expression in subpopulations of mouse thymocytes. Single-cell suspension from the thymuses of 5-wk-old mice (BALB/c and MRL-*lpr/lpr*) was prepared, and 10^6 cells were analyzed by three-color flow cytometry on a FACScan® as described in Materials and Methods. The panels at left are two-dimensional representations of mouse thymocytes for CD4 and CD8 expression. The expression of the Fas antigen was examined

for 5,000 cells of the thymocyte subsets, CD4⁺CD8⁺ (DP), CD4⁺CD8⁻ (CD4 SP), CD4⁻CD8⁺ (CD8 SP), and CD4⁻CD8⁻ (DN), and is shown in the eight panels at right. (b) Expression of Fas in DN thymocytes. Mouse thymocytes were incubated with PE-conjugated anti-CD4 and anti-CD8, FITC-conjugated anti-CD3 and biotinylated anti-Fas antibody (Jo2), which was followed by incubation with streptavidin-TRI-COLOR. The DN thymocytes (CD4⁻CD8⁻) were divided into CD3⁻ (R1 region) or CD3⁺ (R2 region) population (left), and the Fas expression in each population was examined (right).

with streptavidin-conjugated TRI-COLOR at 4°C for 30 min. The cells were then washed twice with staining solution, suspended in 400 μ l of the same solution containing 5 μ g/ml of propidium iodide, and analyzed by flow cytometry. Three-color flow cytometry was performed on a FACScan® (Becton Dickinson) equipped with a 488-nm argon laser. Data from 5,000 or more cells were collected and analyzed on a FACScan® using LYSIS II software.

Cell Killing Assay. Thymocytes (2×10^6 cells) were incubated for various periods at 37°C with the purified Jo2 antibody in 0.5 ml of RPMI 1640 medium containing 10% FCS. The cells were washed once with RPMI 1640 medium, and the dead cells were quantified by flow cytometry after staining with 5 μ g/ml propidium iodide. To examine the DNA degradation, chromosomal DNA was prepared from the thymocytes using a SepaGene kit from Sanko Junyaku (Tokyo, Japan), and analyzed on an agarose gel in Tris-borate buffer (17).

Results

Expression of Fas in Mouse Thymocytes. We previously established a hamster hybridoma (Jo2) producing anti-mouse Fas monoclonal antibody, and showed that more than 95% of mouse thymocytes abundantly express Fas (8). In contrast, Fas expression was not observed in thymocytes from *lpr*-mice (MRL/Mp-*lpr/lpr*). To examine the Fas expression in mouse thymocytes in more detail, thymocytes from BALB/c-+/+ and MRL-*lpr/lpr* were analyzed by three-color flow cytometry using antibodies against CD4, CD8 and Fas. As shown in Fig. 1, thymocytes from 5-wk-old wild-type and *lpr* mice had virtually the same composition of CD4⁻CD8⁻ double negative cells (DN), CD4⁺CD8⁺ DP, and CD4⁺CD8⁻ or CD4⁻CD8⁺ single positive cells (SP), confirming that the *lpr* mutation does not cause the apparent abnormality in thymocyte development (18, 19). Each subpopulation of thymocytes was then examined for the Fas expression. Double and single positive thymocytes from the wild-type mice uni-

formly express Fas, and its intensity was almost identical between the two populations. On the other hand, DN thymocytes hardly expressed Fas except for a minor subpopulation. This Fas-positive CD4⁻CD8⁻ subpopulation was found to be CD3 positive (Fig. 1c). On the contrary, in accordance with the little expression of the functional Fas mRNA in the thymus of *lpr* mice (13), no thymocytes from *lpr* mice expressed Fas on their surface.

Cytotoxicity of Anti-Fas Antibody against Thymocytes. The anti-Fas antibody Jo2 causes apoptosis in mouse WR19L cell transformants expressing Fas (8). To examine whether this antibody has cytotoxic activity on mouse thymocytes, thymocytes were incubated with various concentrations of the purified anti-Fas antibody at 37°C for 16 h in the presence of 30 μ g/ml of cycloheximide. As shown in Fig. 2a, the thymocytes from wild-type mice were killed by anti-Fas antibody in a dose-dependent manner. On the other hand, the antibody did not have any effect on the thymocytes from CBA-*lpr^{cg/lpr^{cg}}*, which express nonfunctional Fas on the cell surface (13). Fig. 2b shows the kinetics of the thymocyte death process by anti-Fas antibody. The thymocytes from the wild-type mice were rapidly killed by anti-Fas antibody, and ~80% of them were dead after a 24-h incubation with 1.0 μ g/ml of the anti-Fas antibody. On the other hand, when the thymocytes from CBA-*lpr^{cg/lpr^{cg}}* were incubated with anti-Fas antibody, most cells were still alive under the same conditions. To confirm that the death of the thymocytes induced by anti-Fas antibody occurred by apoptosis, chromosomal DNA was prepared from the thymocytes after incubation with anti-Fas antibody, and analyzed by agarose gel electrophoresis. As shown in Fig. 3, chromosomal DNA was fragmented after a 3-h incubation with anti-Fas antibody. After 6 h, most of the chromosomal DNA was degraded in the step ladder fashion that is characteristic of apoptosis. As a control, thymocytes from the wild-type mice were treated for 6 h with the anti-

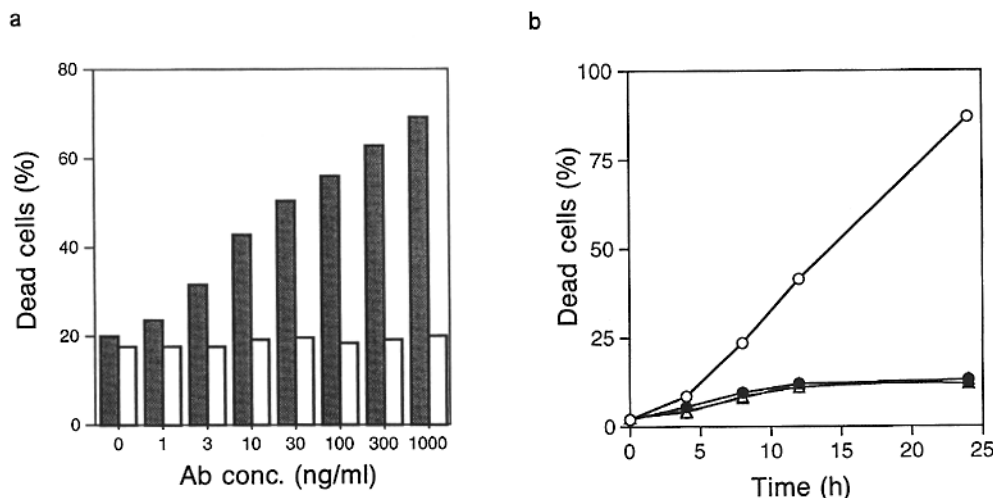


Figure 2. The cytotoxicity of anti-Fas antibody to mouse thymocytes. (a) The dose-dependent cytotoxicity of the anti-Fas antibody. Thymocytes (10^6 cells) from BALB/c (shaded bars) or CBA-*lpr^{cg/lpr^{cg}}* (open bars) were incubated at 37°C for 16 h with various concentrations of purified anti-Fas antibody (1 ng/ml to 1 μ g/ml) in the presence of 30 μ g/ml of cycloheximide. Thereafter, the percentage of dead cells was quantified by FACS® analysis after staining with propidium iodide. (b) Kinetics of the killing process of the thymocytes. Thymocytes from BALB/c-+/+ (○, ●) and CBA-*lpr^{cg/lpr^{cg}}* (Δ, ▲) were incubated at 37°C for the indicated periods with (○, Δ) or without (●, ▲) 1 μ g/ml of anti-Fas antibody, and the percentage of dead cells was determined as described above.

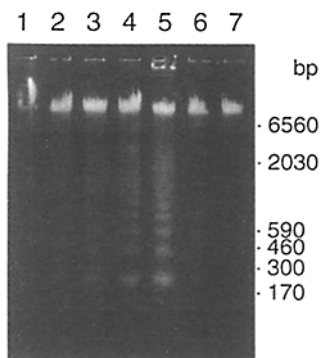


Figure 3. Fragmentation of thymic chromosomal DNA by anti-Fas antibody. Thymocytes (2×10^6 cells) from BALB/c mice were incubated at 37°C in 0.5 ml of RPMI medium containing $1 \mu\text{g/ml}$ of the anti-Fas antibody and $30 \mu\text{g/ml}$ cycloheximide (lanes 1–5). At 0 (lane 1), 1.5 (lane 2), 3.0 (lane 3), 4.5 (lane 4), and 6.0 h (lane 5), the chromosomal DNA was analyzed by electrophoresis on a 1.5% agarose gel. Chromosomal DNA from the thymocytes incubated for 6 h without anti-Fas antibody (lane 6) or with $1 \mu\text{g/ml}$ of the anti-CD5 antibody (lane 7) in the presence of $30 \mu\text{g/ml}$ cycloheximide was also analyzed.

CD5 antibody. This treatment did not cause any degradation of chromosomal DNA (Fig. 3).

Apoptosis of thymocytes can be induced by various agents such as glucocorticoids or thymocyte activators (20–24). To characterize the apoptosis of thymocytes induced by anti-Fas antibody, and to distinguish it from apoptosis induced by other agents, the thymocytes were incubated for 18 h with either anti-Fas antibody, dexamethasone or a combination of PMA and A23187 ionomycin. Both dexamethasone and PMA/ionomycin rapidly induced apoptosis of the thymocytes, and about 80% of them were dead after 18 h (Fig. 4). Dexamethasone or PMA/ionomycin also killed the thymocytes from *lpr^g/lpr^g* mice as efficiently as the wild-type thymocytes, but Jo2 did not induce apoptosis in the Fas-defective *lpr^g/lpr^g* strain (Fig. 4). Furthermore, as previously reported (20–24), the apoptosis induced by dexamethasone or PMA/ionomycin was almost completely inhibited by cycloheximide, whereas it enhanced the apoptosis induced by anti-Fas antibody.

DP but Not SP Thymocytes Are Sensitive to Fas-Induced Apoptosis. As shown above, Fas is expressed in most thymocytes except for those with the CD4⁺CD8[−]CD3[−] phenotype. To examine which subset of thymocytes are sensitive to the cytotoxic activity of Jo2 antibody, thymocytes were incubated

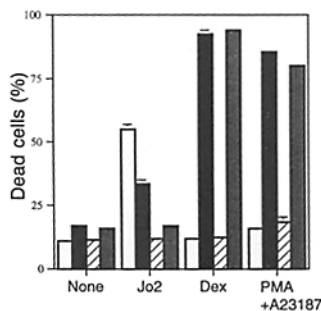


Figure 4. Characterization of apoptosis of the thymocytes induced by various agents. Thymocytes from CBA^{+/+} (filled and open bars) or CBA^{lpr^g/lpr^g} (slashed and shaded bars) were incubated at 37°C for 18 h with $1.0 \mu\text{g/ml}$ of anti-Fas antibody (Jo2), $1.0 \mu\text{M}$ dexamethasone (Dex) or a mixture of 10 ng/ml of PMA and $0.5 \mu\text{M}$ ionomycin in the presence (open and slashed bars) or absence (filled and shaded bars) of $30 \mu\text{g/ml}$ of cycloheximide.

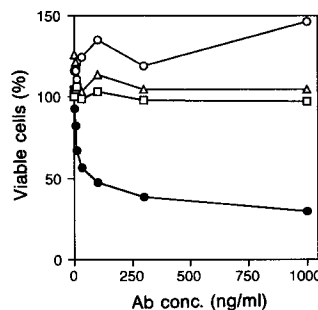


Figure 5. Selective killing of CD4⁺CD8⁺ thymocytes by anti-Fas antibody. Thymocytes (2×10^6 cells) from BALB/c mice were incubated at 37°C for 16 h with various concentrations of anti-Fas antibody in the presence of 30 mg/ml of cycloheximide. After incubation, viable thymocytes were analyzed by two-color flow cytometry for CD4 and CD8 expression. The cell number of each subpopulation of the thymocytes, CD4⁺CD8⁺ (●), CD4⁺CD8[−] (Δ), CD4[−]CD8[−] (□), after incubation with anti-Fas antibody is expressed as a percentage of that of the thymocytes incubated without anti-Fas antibody.

at 37°C for 16 h with various concentrations of Jo2 antibody in the presence of $30 \mu\text{g/ml}$ of cycloheximide, and the viable cells were analyzed by flow cytometry using anti-CD4 and CD8 antibody. As shown in Fig. 5, the DP thymocytes were sensitive to the cytotoxic activity of the Jo2 antibody, and more than half of the DP thymocytes were killed within 16 h by the antibody at a concentration of 100 ng/ml . On the other hand, the viability of either CD4⁺CD8[−] or CD4[−]CD8⁺ SP cells did not change after incubation with $1 \mu\text{g/ml}$ anti-Fas antibody. These results indicated that the SP thymocytes are resistant to Fas-induced apoptosis, although they express Fas as abundantly as CD4⁺CD8⁺ DP thymocytes.

In Vivo Apoptosis of Thymocytes by Anti-Fas Antibody. We showed that mice given an intraperitoneal injection of anti-Fas antibody rapidly died (8). Although this administration caused acute hepatic failure, we could not detect any histological abnormality in the thymus 2 h after injection of the antibody. Since the thymocytes were sensitive to the cytotoxic activity of the antibody in vitro, we reexamined the in vivo effect of the antibody on the thymus. As previously found, when $100 \mu\text{g}$ of the purified Jo2 antibody was intraperitoneally injected into the wild-type mice, most of them died within 3 h, but some survived for 6–7 h (8). Therefore, the thymus was removed at various intervals after injection with Jo2 antibody, and its chromosomal DNA was analyzed on an agarose gel electrophoresis. As shown in Fig. 6, the thymic DNA was partially fragmented at 3 h after injection

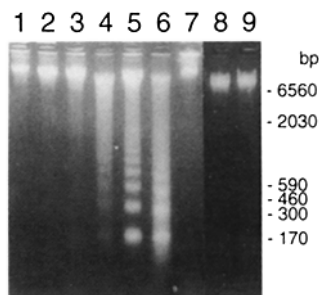


Figure 6. Apoptosis in the thymus induced by in vivo administration of anti-Fas antibody. 3-wk-old BALB/c mice (lanes 1–6) or MRL^{lpr/lpr} mice (lane 8) were injected with $100 \mu\text{g}$ of Jo2 antibody. Thymic DNAs were prepared at 0 (lane 1), 0.5 (lane 2), 1.0 (lane 3), 2.0 (lane 4), 3.0 (lane 5), or 5.0 h (lane 6) after injection of the Jo2 anti-Fas antibody. DNAs were analyzed by electrophoresis on a 1% agarose gel in TBE buffer containing $0.5 \mu\text{g/ml}$ of ethidium bromide. The thymic DNA prepared from the wild-type mice at 5 h after injection of the same amount of the control hamster IgG (lane 7) or anti-CD5 antibody (lane 9) was also analyzed.

with the antibody, and most DNA was degraded in a step ladder fashion after 5 h. On the other hand, when Jo2 antibody (100 μ g) was injected into *lpr* mice, or anti-CD5 antibody (100 μ g) was injected into the wild-type mice, no degradation of thymic chromosomal DNA was observed (Fig. 6). These results indicated that the thymocytes are specifically sensitive in vivo to the apoptosis-inducing activity of the anti-Fas antibody, although it takes more time than apoptosis in the liver, which occurs within 2 h (8).

Discussion

Recently, Drappa et al. (25), reported that most mouse thymocytes, including CD4⁺CD8⁻ cells, express Fas with the highest levels being in CD4⁺CD8⁺ thymocytes. Here, we examined Fas expression using three color method, and confirmed its expression in most thymocytes. However, unlike the report by Drappa et al. (25), the most T-precursor cells (CD4⁺CD8⁻CD3⁻) did not express Fas, and little difference in the Fas expression levels was detected between DP and SP thymocytes. Our results suggest that the Fas expression is induced at the stage from CD3⁻CD4^{lo}CD8^{lo} to CD3^{lo}CD4⁺CD8⁺ and is thereafter expressed throughout the thymic development of mouse thymocytes. This distribution of Fas in mouse thymocytes is significantly different from that in human thymocytes (26, 27), where Fas or APO-1 is not expressed (26), or expressed weakly in CD4⁺CD8⁻ DN and CD4⁺CD8⁺ DP thymocytes, but not in CD4⁺CD8⁻ or CD4⁺CD8⁺ SP cells (27).

Since the loss-of-function mutations of Fas or Fas ligand causes lymphadenopathy and autoimmune disease (13, 14), it is likely that the Fas system is involved in the apoptotic process during the development, maturation, or turnover of T cells. Here, we showed that the peritoneal administration of anti-Fas antibody into mice caused massive apoptosis in thymocytes. Since most thymocytes are comprised of CD4⁺CD8⁺ DP cells, this agreed with the results obtained in vitro, which showed that the DP thymocytes are selectively sensitive to the cytolytic activity of the anti-Fas antibody. These results suggest a role of Fas in the thymic T cell development, since apoptosis of thymocytes mainly occurs in the DP stage (28). However, the Fas-induced apoptosis is significantly different from the apoptosis induced by activation of thymocytes, which may or may not represent negative selection. The apoptosis induced by activation of thymocytes by PMA and ionomycin was blocked by inhibitors of protein or RNA synthesis, whereas Fas-induced apoptosis was enhanced by cycloheximide (Fig. 4). Furthermore, thymocytes from *lpr*-mice which do not express Fas can be killed by activation with PMA and ionomycin, or anti-CD3 antibody (29,

30). Negative selection occurs when T cell receptors of immature CD4⁺CD8⁺ cells are engaged by exposure to self-antigen peptides complexed with self-MHC on thymic stroma cells (3). Apoptosis of DP cells by anti-CD3 antibody was therefore proposed to reflect a model of negative selection (24, 31). However, this model does not explain the negative selection observed in transgenic mice overexpressing Bcl-2 (32, 33). In these mice, DP thymocytes are resistant against the anti-CD3-induced apoptosis in vitro, yet negative selection is not affected or only marginally disturbed in vivo. The Fas-mediated apoptosis is only partially inhibited by Bcl-2 (34), and *lpr* or *gld* thymocytes undergo normal negative selection (35). Taken together, there may be two apoptotic mechanisms for negative selection induced by TCR engagement; one pathway is sensitive to Bcl-2, while the other is resistant to Bcl-2, and Fas may be involved in the latter pathway. In addition to the apoptosis that occurs during negative selection, thymocytes which do not undergo positive selection die by apoptosis (programmed cell death) (1). Whether or not the Fas system is involved in this process remains to be elucidated.

As found in human naive mature T cells (36, 37), CD4⁺ and CD8⁺ SP thymocytes could not be killed by anti-Fas antibody despite their high expression of Fas. These cells may be devoid of the apoptotic signal transduction system through Fas, or they express proteins that inhibit Fas-mediated apoptosis. One candidate for such inhibitory protein is Bcl-2 (38) which is abundantly expressed in SP thymocytes, but hardly expressed in DP thymocytes (39). However, the overexpression of Bcl-2 only partially inhibits the Fas-mediated apoptosis in various cell types (34). Since several Bcl-2-related proteins have been identified (40, 41), it would be of interest to study whether they modulate Fas-mediated apoptosis.

Recently, Alderson et al. (42) have reported that costimulation of human thymocytes with anti-Fas antibody and anti-CD3 causes the Fas-dependent proliferation of thymocytes. With mouse thymocytes, we detected only the cytolytic activity of the anti-Fas antibody in the absence of anti-CD3 antibody, and not the growth-promoting activity. Natural Fas ligand also caused only apoptosis in mouse thymocytes (our unpublished results). It is possible that human and mouse thymocytes respond differently to the anti-Fas antibody or Fas ligand, or that stimulation of the TCR modified the Fas-mediated signal transduction system in the thymocytes. However, it is more likely that the enhancement of the CD3-induced proliferation of human thymocytes by the anti-Fas antibody is due to the inhibition of the anti-CD3-induced apoptosis of mature thymocytes (such as CD4⁺ or CD8⁺ SP thymocytes) by the anti-Fas antibody, and expansion of the rescued cells with anti-CD3 antibody.

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