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Overexpressed Bcl-x_L prevents bacterial superantigen-induced apoptosis of thymocytes *in vitro*

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Abstract

***bcl-x*, a homologous gene of *bcl-2*, has an anti-apoptotic function and appears to play a critical role in the development of lymphoid systems. To investigate the effect of over-expressed Bcl-x_L on the development of T lymphocytes, we established two lines of transgenic mice by using Eμ-chicken *bcl-*x_L (*cbcl-*x_L) transgene, where the cBcl-x_L protein was expressed mainly in lymphoid cells. Although thymocytes and splenocytes from *cbcl-*x_L transgenic mice are resistant to apoptosis *in vitro*, clonal deletion of thymocytes, recognizing endogenous self-superantigens in the thymus, still normally proceeded and no self-reactive T cells were found in the spleen of the transgenic mice. To dissect clonal deletion, we utilized two *in vitro* models, thymocytes/antigen presenting cells co-culture system and fetal thymus organ culture system. In both, bacterial superantigen *staphylococcus aureus* enterotoxin B (SEB) induces apoptosis of T cells with Vβ8$^+$ T cell receptor (TCR) reacting to SEB, which mimics clonal deletion of self-reactive thymocytes *in vivo*. SEB-induced depletion of Vβ8$^+$ T cells from thymocytes when taken from the transgenic mice was effectively inhibited. The data might raise the possibility that cell death process involved in clonal deletion in the thymus is a form of apoptosis inhibited by Bcl-x_L.**

Keywords: thymocytes; clonal deletion; bcl-x_L; transgenic mouse; superantigen

Abbreviations: SEB, *staphylococcus aureus* enterotoxin B; TCR, T cell receptor; MHC, major histocompatibility complex; PI, propidium iodide; APCs, antigen presenting cells; ABC avidin-biotin-peroxidase complex; HRP, horseradish peroxidase; MAbs, monoclonal antibodies; FBS, fetal bovine serum

Introduction

In immune systems, elimination of a certain population of lymphocytes is indispensable for maintaining homeostasis and removing harmful cells, namely the elimination of nonfunctional and self-reactive immune cells generated during their development and of immune cells after completing immune responses. Depletion of self-reactive immature T cells in the thymus (clonal deletion or thymic negative selection) is triggered by a strong association between T cell receptor (TCR) and complex molecules consisting of self-antigens and major histocompatibility complex (MHC) expressed on thymic stroma cells (Blackman *et al*, 1990; Nossal, 1994). Direct involvement of the cell death process in clonal deletion in the thymus has been supported by previous observations: strong crosslinking of CD3–TCR complex, which is thought to mimic the process of recognition of self-antigens by TCR, induces apoptotic cell death of thymocytes (Harvan *et al*, 1987), and administration of a peptide antigen into transgenic mice expressing TCR that reacts to the antigen results in deletion of immature thymocytes through apoptotic cell death (Murphy *et al*, 1990).

Apoptosis is a mechanism of cell death which largely accounts for physiological cell death to remove unwanted cells and plays an important role in a variety of biological events, including programmed cell death during embryogenesis. Apoptotic cell death is negatively regulated by anti-apoptotic genes including the *bcl-2* family (for review see Cory, 1995). Protooncogene *bcl-2* and its relative, *bcl-x*, are expressed in a variety of tissues including thymus (Hockenbery *et al*, 1991; Krajewski *et al*, 1994), providing a naive assumption of their involvement in the selection of thymocytes. *bcl-x* encodes two proteins through an alternative splicing of the mRNA, Bcl-x_L which inhibits apoptosis like Bcl-2, and Bcl-x_S which counteracts the anti-apoptotic activities of Bcl-2 and Bcl-x_L (Boise *et al*, 1993). mRNA for Bcl-x_L is present in various tissues of human and mouse, whereas mRNA for Bcl-x_S appears to be absent in mouse tissues although significant levels of mRNA for Bcl-x_S is present in human tissues (Boise *et al*, 1993; Garcia *et al*, 1994). Immunohistochemical analysis showed the topographical difference of expression between Bcl-2 and Bcl-x_L in the thymus: Bcl-x_L positive cells exist predominantly in the cortex, whereas Bcl-2 positive cells in the medulla (Hockenbery *et al*, 1991; Krajewski *et al*, 1994). This observation suggests that the expression levels of Bcl-2 and Bcl-x_L are strictly regulated through T cell development and that both exert different physiological roles. Consistently, gene targeting studies indicated that *bcl-2* promotes the survival of mature T cells rather than of immature T cells (Veis *et al*, 1993; Nakayama *et al*, 1993,

1994; Kamada *et al*, 1995), whereas *bcl-x* plays a dominant role in sustaining immature T cells (Motoyama *et al*, 1995).

The idea of direct involvement of Bcl-2 and Bcl-x_L in the process of positive and negative selections of thymocytes is supported by highly regulated topographical expression of the proteins during thymocyte development. However, it has apparently been argued by studies of transgenic mice expressing Bcl-2 and Bcl-x_L in thymocytes, in which self-reactive T cells are still normally eliminated and absent in periphery (Sentman *et al*, 1991; Strasser *et al*, 1991; Grillot *et al*, 1995), although slight but significant protective effects by Bcl-2 in clonal deletion in the thymus have also been described (Strasser *et al*, 1991; Siegel *et al*, 1992). These observations suggested the existence of forms of apoptotic cell death which are not prevented by Bcl-2 family proteins. Since clonal deletion might be a complex process, in order to dissect the process in simpler systems, we have generated transgenic mice overexpressing cBcl-x_L in lymphoid cells and analyzed the effects of the over-expressed cBcl-x_L on clonal deletion of thymocytes *in vivo* and also in *in vitro* systems mimicking clonal deletion.

Here, we have shown that overexpressed cBcl-x_L did not prevent superantigen-induced depletion of a subset of thymocytes *in vivo* but prevented bacterial superantigen-induced apoptosis of the thymocytes *in vitro*.

Results

Generation of Eμ-*cbcl-x$_L$* transgenic mice

To develop transgenic mice expressing cBcl-x_L in lymphoid cells, we used a transgene construct in which *cbcl-x*$_L$ expression is driven by Eμ enhancer and SV40 promoter (Figure 1). Among five founder mice generated, we established two independent transgenic lines (designated line 17 and 31). Western blot analysis using antibodies specific for cBcl-x_L protein revealed that cBcl-x_L expression was restricted to the lymphoid organs, thymus and spleen in two lines (Figure 2a). In line 17, which carries about 20 copies of the transgene, cBcl-x_L was detected predominantly in the thymus, and in line 31 with about two copies of the transgene, the expression level of cBcl-x_L was higher in the spleen than in the thymus (Figure 2b). As shown in Figure 2c, immuno-fluorescence microscopic study of thymic sections revealed that cBcl-x_L protein was evenly present at both medulla and cortex of thymus from *cbcl-x*$_L$ transgenic mice (data not shown for line 31).

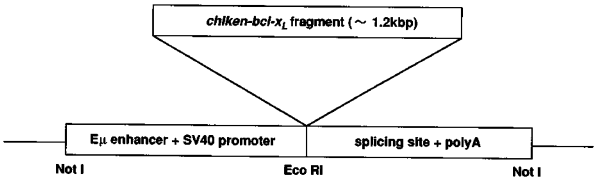

Figure 1 Eμ-*cbcl-x*$_L$ transgene construct. *cbcl-x*$_L$ cDNA fragment of 1.2 kb containing the entire coding region for cBcl-x_L protein was inserted into the EcoRI site of the Eμ-SV40 cassette. The NotI 3.0 kb fragment was used for microinjection.

Improved protection of *cbcl-x$_L$* transgenic lymphocytes from apoptotic stimuli

To confirm that overexpressed cBcl-x_L in lymphocytes exerts anti-apoptotic ability, thymocytes and splenocytes from *cbcl-x*$_L$ transgenic mice and their non-transgenic littermates were cultured *in vitro* in RPMI 1640 medium supplemented with 10% FBS. The extent of apoptotic cell death was assessed by quantifying the subdiploid fraction using flow cytometry with propidium iodide (PI) staining. As shown in Figure 3a and b, splenocytes and thymocytes from the *cbl-x*$_L$ transgenic lines 17 and 31 revealed improved survival than those from their

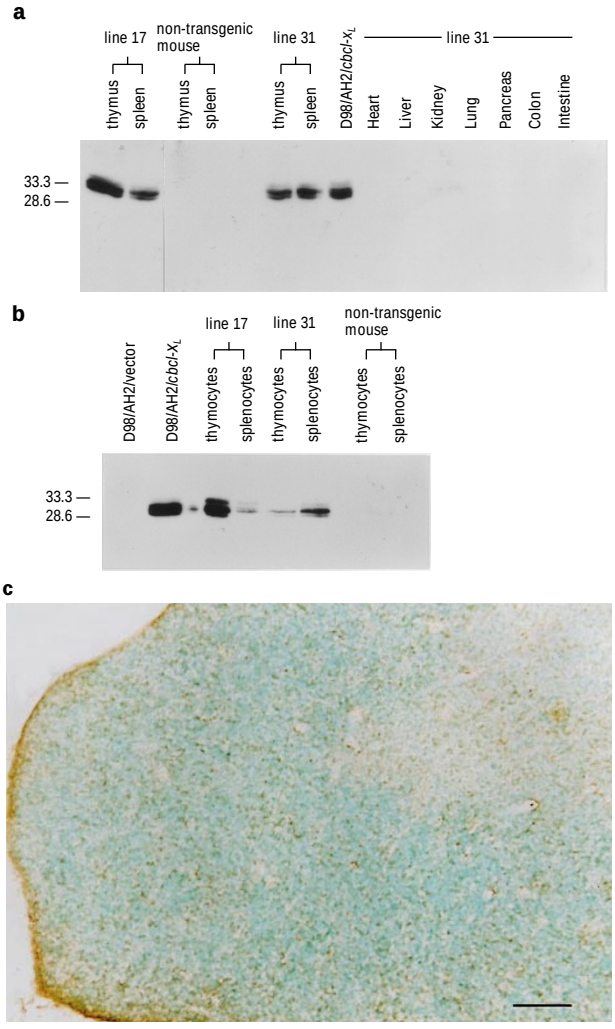

Figure 2 Expression of cBcl-x_L in transgenic mice. (a, b) Western blot analysis of cBcl-x_L expression. Total proteins were extracted from various tissues of 6-week old *cbcl-x*$_L$ transgenic line 17, 31 and non-transgenic littermate of line 17. Five μg (a) and 2 μg (b) each of the total proteins were subjected to Western blot analysis. cBcl-x_L protein was detected with anti-cBcl-x_L polyclonal antibody which is specific to cBcl-x_L and does not react to mouse Bcl-x_L. Total proteins extracted from derivatives of D98/AH2 cell line expressing and not expressing cBcl-x_L were used as positive and negative controls, respectively. Molecular size standards are shown at left in kDa. (c) Immunohistochemical detection of cBcl-x_L protein in thymus of *cbcl-x*$_L$ transgenic line 17. Thymic sections were immunostained with anti-cBcl-x_L antibodies as described in Materials and Methods (Bar=100 μm).

non-transgenic littermates. Thymocytes from the *cbl-x_L* transgenic lines 17 and 31 also showed higher resistance to dexamethasone-induced apoptosis (Figure 3c). Thus, cBcl-x_L expressed from the transgene is able to protect thymocytes and splenocytes from apoptosis *in vitro*.

Overexpressed cBcl-x_L, however, did not affect cellularity in the thymus and spleen, except in *cbcl-x_L* transgenic line 31, which consistently showed more splenocytes (1.5 ×) than non-transgenic littermates. Ratios of T cell subsets ($CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^-CD8^-$) in the thymus was also not affected by overexpressed cBcl-x_L (data not shown).

Failure of overexpressed cBcl-x_L to inhibit clonal deletion induced by superantigens *in vivo*

Since clonal deletion of self-reactive T cells is thought to proceed by apoptotic cell death in the thymus (Harvan *et al*, 1987; Murphy *et al*, 1990), we examined whether self-reactive T cells escaped clonal deletion and existed in the spleen of *cbcl-x_L* transgenic mice. Mouse endogenous superantigen Mls-2^a causes clonal deletions of Vβ3$^+$ T cells in association with I-E molecules (Pullen *et al*, 1988). We crossed C57BL/6 (I-E^- and Mls-2^b):*cbcl-x_L* transgenic line 17 with DBA/2 (I-E^+ and Mls-2^a) to obtain F1 (BDF1) *cbcl-x_L* transgenic mice, and studied the protective effect of overexpressed cBcl-x_L on clonal deletion of Vβ3$^+$T cells reacting with the superantigen Mls-2^a. As shown in Figure 4 and Table 1, the number of T cells highly expressing Vβ3$^+$ were reduced equally in both the spleens and thymi of the BDF1 *cbcl-x_L* transgenic mice and of the BDF1 non-transgenic littermates. These results showed that, in spite of enhanced resistance of cBcl-x_L-overexpressing thymocytes to apoptosis *in vitro*, clonal deletion *in vivo* is not significantly affected by overexpressed cBcl-x_L, consistent with the previous observations (Sentman *et al*, 1991; Strasser *et al*, 1991; Grillot *et al*, 1995).

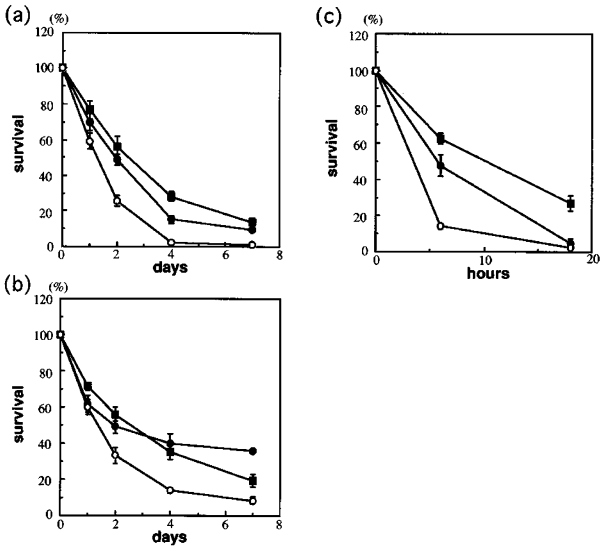

Figure 3 Extended survival of thymocytes and splenocytes *in vitro* by overexpressed cbcl-x_L. Thymocytes (a, c) and splenocytes (b) from 6-week old *cbcl-x_L* transgenic line 17 (closed square), line 31 (closed circle) and non-transgenic littermates of line 31 (open circle) were cultured at 5×10^5 cells/ml in RPMI 1640 medium supplemented with 10% FBS at 37°C in the absence (a, b) and presence (c) of 0.5 μM dexamethasone. At the indicated times, cells were harvested and their viability was examined by flow cytometry after staining with PI as described in Material and Methods. Results are expressed as mean ± SD of values obtained from four and five independent experiments with line 31 (transgenic and non-transgenic) and with line 17, respectively.

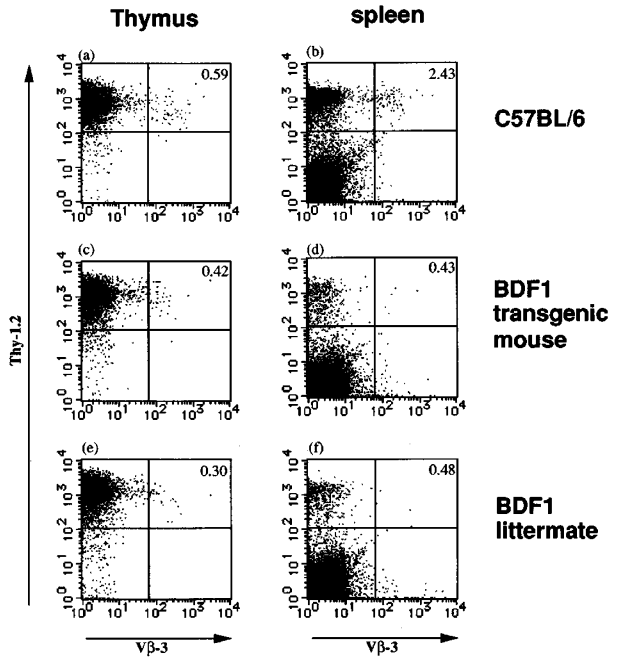

Figure 4 Failure of overexpressed cBcl-x_L to inhibit clonal deletion induced by endogenous superantigens *in vivo*. Thymocytes (a, c, e) and splenocytes (b, d, f) from 5-week old C57BL/6 (a, b), and *cbcl-x_L* transgenic mouse (c, d) and non-transgenic littermate (e, f) of BDF1 (DBA/2 x *cbcl-x_L* transgenic line 17) were double-stained with anti-Thy-1.2-FITC and anti-Vβ3-PE MAbs. Representative data of several independent experiments showing superimposable results are shown. Numbers in boxes indicate the percentage of Vβ3$^+$ cells among Thy-1.2$^+$ cells.

Table 1 Failure of overexpressed cBcl-x_L to inhibit clonal deletion induced by endogenous superantigen Mls

	Percentage of Vβ3$^+$ in Thy 1.2$^+$ cells			
	Thymus		Spleen	
C57BL/6	0.63 ± 0.05	(*n*=5)	2.87 ± 0.53	(*n*=5)
BDF1 (DBA/2 x line 31)				
cbcl-x_L +	0.33 ± 0.04	(*n*=3)	0.32 ± 0.17	(*n*=4)
cbcl-x_L −	0.36 ± 0.03	(*n*=3)	0.33 ± 0.06	(*n*=4)
BDF1 (DBA/2 x line 17)				
cbcl-x_L +	0.27 ± 0.11	(*n*=5)	0.38 ± 0.08	(*n*=4)
cbcl-x_L −	0.24 ± 0.06	(*n*=5)	0.40 ± 0.13	(*n*=4)

F1 mice carrying *cbcl-x_L* transgene, I-E^+ and Mls-2^a were obtained by crossing *cbcl-x_L* transgenic mice with DBA/2 mice. Thymocytes and splenocytes from 5 week-old mice were double-stained with anti-Thy-1.2-FITC and anti-Vβ3-PE MAbs. Results were expressed as a percentage (mean ± SD of values obtained from *n* animals) of Vβ3$^+$ cells among Thy-1.2$^+$ cells. There was no statistically significant difference in the percentage of Vb3$^+$ cells between transgenic and non-transgenic mice by Student's t test.

Prevention of deletion of thymocytes reacting with bacterial superantigen *in vitro* by overexpressed cBcl-x_L

Clonal deletion *in vivo*, which overexpressed cBcl-x_L was not able to inhibit, is conceivably a complex process involving not only apoptotic cell death but also different mechanisms of eliminating self-reactive T cells. To understand whether or not this apoptotic cell death process involved in clonal deletion is inhibitable by overexpressed cBcl-x_L, we utilized two *in vitro* systems which closely mimic the apoptotic process of clonal deletion (Jenkinson *et al*, 1989; Aiba *et al*, 1994). One was a co-culture system of thymocytes with antigen presenting cells (APCs), and the other was a fetal thymus organ culture system. In both systems, bacterial superantigen *staphylococcus aureus* enterotoxin B (SEB) which causes specific depletion of Vβ8$^+$ T cells through apoptosis was used in place of self-antigens (White *et al*, 1989).

In a co-culture system, a B lymphoma cell line, A20.2J, which expresses MHC class II, I-E molecules at a high level, was employed as APCs. Thymocytes from *cbcl-x*$_L$ transgenic line 17 and non-transgenic littermates were individually co-cultured with mitomycin C-treated A20.2J in both the presence and absence of SEB for 24 to 30 h as described in Materials and Methods. The flow cytometric analysis revealed that while depletion of Vβ8$^+$ T cells from non-transgenic thymocytes occurred, a considerable fraction of Vβ8$^+$ T cells in transgenic thymocytes remained undeleted (Figure 5A, Table 2). There was no difference in the number of SEB-non reactive Vβ6$^+$ T cells between

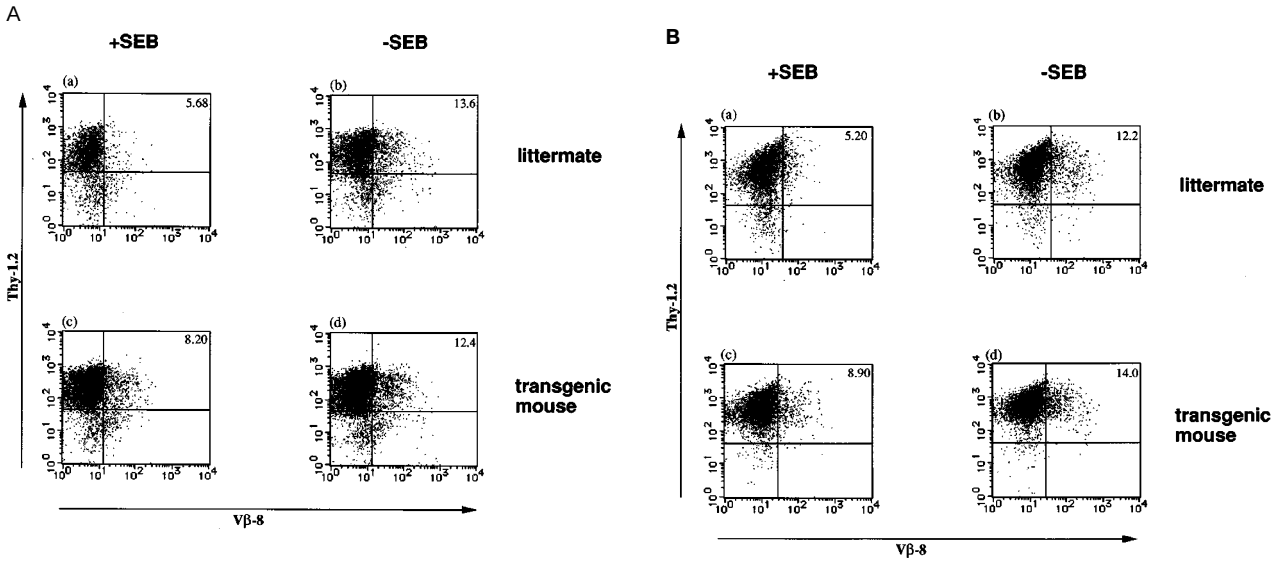

Figure 5 Inhibition of SEB-induced depletion of Vβ8$^+$ thymocytes *in vitro* by overexpressed cBcl-x_L. (A) Thymocytes from 4 to 6-week old *cbcl-x*$_L$ transgenic line 17 (c, d) and their non-transgenic littermates (a, b) were co-cultured with mitomycin C-treated A20.2J in the presence (a, c) and absence (b, d) of SEB. After 24 h, cells were harvested by extensive pipetting and double-stained with anti-Vβ8-FITC and anti-Thy-1.2-PE MAbs. Representative data of three independent experiments showing superimposable results are shown. Numbers in boxes indicate the percentage of Vβ8$^+$ cells among Thy-1.2$^+$ cells. (B) Thymi of E14 embryos from *cbcl-x*$_L$ transgenic line 17 (c, d) and non-transgenic littermate (a, b) were organ-cultured for 10 days. Cells were incubated with SEB for 18 h, harvested and double-stained with anti-Vβ8-FITC and anti-Thy-1.2-PE MAbs. Representative data of three independent experiments showing superimposable results are shown. Numbers in boxes indicate the percentage of Vβ8$^+$ cells among Thy-1.2$^+$ cells.

Table 2 Inhibition of SEB-induced deletion of Vβ8$^+$ thymocytes by overexpressed cBcl-x_L and hBcl-2 in co-culture system

Exp.	Transgene	Percentage of VB8$^+$ in Thy-1.2$^+$ cells SEB +	SEB –	Incubation time with SEB	SEB-induced apoptosis of Vβ8$^+$ cells (%)
I	*cbcl-x*$_L$	8.19±0.48 (*n*=5)	11.9±0.35 (*n*=5)	24 h	31.2
	–	4.59±0.65 (*n*=5)	14.2±0.84 (*n*=5)		67.6
II	*cbcl-x*$_L$	8.54±0.55 (*n*=5)	11.7±0.36 (*n*=5)	30 h	26.9
	–	6.12±0.79 (*n*=4)	15.8±1.66 (*n*=4)		61.1
III	*cbcl-x*$_L$	6.53±0.43 (*n*=5)	10.2±0.62 (*n*=5)	24 h	34.8
	–	4.18±0.75 (*n*=3)	9.26±0.92 (*n*=3)		54.9
IV	*hbcl-2*	7.91±1.08 (*n*=5)	10.3±1.02 (*n*=5)	30 h	23.2
	–	4.96±0.39 (*n*=5)	9.87±1.40 (*n*=5)		49.7

Thymocytes from 4 to 6 weeks old *cbcl-x*$_L$ transgenic mice and their non-transgenic littermates were individually co-cultured with A20.2J in both the presence and absence of SEB for the indicated time, and double-stained with anti-Vβ8-FITC and anti-Thy-1.2-PE MAbs. Results are expressed as the percentage (mean±SD of values from n independent experiments) of Vβ8$^+$ cells among Thy-1.2$^+$ cells. ANOVA and Sheffe's test revealed statistically significant difference in the percentage of Vβ8$^+$ cells among Thy-1.2$^+$ cells between SEB$^+$/transgenic and SEB$^+$/non-transgenic mice.

Table 3 Inhibition of SEB-induced deletion of Vβ8$^+$ thymocytes by overexpressed cBcl-x_L in *in vitro* fetal thymic organ culture

Exp.	Transgene	Percentage of Vβ8$^+$ in Thy-1.2$^+$ cells SEB +	SEB –	SEB-induced apoptosis of Vβ8$^+$ cells (%)	Percentage of Vβ6 in Thy-1.2$^+$ cells SEB +	SEB –
I	*cbcl-x$_L$*	11.1 $\pm$ 1.26 (*n*=4)	14.2 (*n*=2)	21.8	ND	
	–	6.18 $\pm$ 0.55 (*n*=4)	14.9 $\pm$ 1.74 (*n*=4)	58.5	ND	
II	*cbcl-x$_L$*	9.00 $\pm$ 0.98 (*n*=4)	13.4 (*n*=2)	32.6	11.2 $\pm$ 1.18 (*n*=3)	11.0 $\pm$ 1.02 (*n*=3)
	–	4.35 $\pm$ 0.94 (*n*=3)	11.8 $\pm$ 0.45 (*n*=4)	63.1	10.9 $\pm$ 1.21 (*n*=3)	12.3 (*n*=2)
II	*cbcl-x$_L$*	14.2 $\pm$ 1.54 (*n*=6)	16.8 (*n*=2)	15.5	8.25 (*n*=2)	8.23 $\pm$ 1.33 (*n*=3)
	–	9.89 $\pm$ 1.85 (*n*=3)	17.4 $\pm$ 0.87 (*n*=3)	43.2	8.83 $\pm$ 0.18 (*n*=3)	8.64 (*n*=2)

Thymi of embryos (E14) from the crosses of Balb/c x C57BL/6 cbcl-x_L transgenic line 17 were organ-cultured for 10 days, and further incubated in the presence and absence of SEB for 18 h. Thymocytes were double-stained with anti-Vβ8-FITC or anti-Vβ6-FITC and anti-Thy-1.2.-PE MAbs. Results are shown as the percentage (mean $\pm$ SD or mean of values from n independent experiments) of Vβ8$^+$ or Vβ6$^+$ cells among Thy-1.2$^+$ cells. ANOVA and Sheffe's test revealed statistically significant difference in the percentage of Vβ8$^+$ cells among Thy-1.2$^+$ cells between SEB$^+$/transgenic and SEB$^+$/non-transgenic mice.

thymocytes from transgenic and non-transgenic mice before and after SEB treatment (data not shown). Similar results were obtained in *bcl-2* transgenic mice (Table 2).

The fetal thymic organ culture system appears to provide an environment resembling that of thymus in mice. SEB is presented as an antigen in a complex with MHC class II I-E on APCs to Vβ8$^+$ thymocytes. We crossed the *cbcl-x$_L$* transgenic line 17 (C57BL/6: I-E$^-$) with BALB/c (I-E$^+$) mice and obtained MHC I-E$^+$ *cbcl-x$_L$* transgenic mouse embryos. From the fetuses (E14), thymic lobes were isolated and cultured for 10 days as described in Materials and Methods. As shown in Figure 5B and Table 3, after an 18 h exposure to SEB, the number of Vβ8$^+$ T cells in the thymic lobes from non-transgenic embryos decreased, whereas the thymic lobes from *cbcl-x$_L$* transgenic embryos contained a significantly larger number of viable Vβ8$^+$ T cells. There was no difference in the number of SEB-nonreactive Vβ6$^+$ T cells between thymocytes from transgenic and non-transgenic embryos before and after SEB treatment (Table 3). Longer incubation with SEB did not significantly reduce the fractions of Vβ6$^+$ and Vβ8$^+$ T cells (data not shown). Similar results were obtained with organ cultures from *bcl-2* transgenic mice (data not shown). These results indicate that deletion of the Vβ8$^+$ T cells induced by SEB is efficiently inhibited by overexpressed cBcl-x_L and Bcl-2.

Discussion

In the present study, we have generated two independent lines of *cbcl-x$_L$* transgenic mice, in which cBcl-x_L expression was targeted to lymphoid cells. Thymocytes and splenocytes from the transgenic mice have shown improved survival compared with those from non-transgenic mice in primary cultures. Thymocytes from both lines were also more resistant to dexamethasone-induced apoptosis. These results indicate that overexpressed cBcl-x_L inhibits apoptosis of lymphocytes.

The result that clonal deletion of T cells with specific TCRβ chains, caused by a mouse endogenous superantigen, proceeded normally even if cBcl-x_L was overexpressed in thymocytes indicates that anti-apoptotic ability of cBcl-x_L had little influence on clonal deletion. This result is consistent with previous observations that in transgenic mice, overexpressed human Bcl-2 and Bcl-x_L in thymocytes are not able to prevent clonal deletion and self-reactive T cells are absent or hardly present in periphery (Sentman *et al*, 1991; Strasser *et al*, 1991; Siegel *et al*, 1992). By using *in vitro* model systems which mimic clonal deletion, we have shown that when taken from the transgenic mice, Vβ8$^+$ thymocytes that recognize SEB resist SEB-induced apoptosis to a significant extent. This result may raise the possibility that apoptotic cell death involved in clonal deletion is a form of cell death which is inhibited by the overexpressed Bcl-2 or Bcl-x_L. This notion is consistent with the previous observation that overexpressed human Bcl-x_L protects thymocytes from anti-CD3 antibody-induced apoptosis *in vivo* (Grillot *et al*, 1995) which is another model of clonal deletion.

There are several possible explanations for the difference between results obtained *in vivo* and *in vitro*. Firstly, the *in vitro* systems might not reflect physiological clonal deletion because the systems are simplified. Stimulation of TCR with SEB *in vitro* might be different from that with self-antigens *in vivo*. Death signals transduced through TCR during clonal deletion might be much stronger than those by SEB *in vitro* or might induce a special form of cell death which is not prevented by anti-apoptotic proteins Bcl-2 and Bcl-x_L. Secondly, some mechanisms different from cell death may be involved in clonal deletion. Rapid engulfment is one possibility, which could take place in spite of prevention of cell death itself by overexpressed Bcl-2 as previously described using neutrophil-*bcl-2* transgenic mice (Lagasse and Weissman, 1994). A recent study using Vβ5 TCR transgenic mice suggested that rapid engulfment of pyknotic cells by F4/80$^-$ Mac-3$^+$ macrophages associates with thymic negative and positive selections (Surh and Sprent, 1994). In *bcl-2* and *bcl-x$_L$* transgenic mice, self-reactive cells may resist apoptosis induced by the association of TCR with self-antigens but these cells may be efficiently engulfed before maturation and may not be detected in peripheral lymphoid tissues. When efficiently removing a large number of thymocytes by positive and negative selections, stimulation of thymocytes by self-antigens might activate not only the cell death process but also a process required for engulfment which is not inhibited by Bcl-2 and Bcl-x_L. Further studies are required to distinguish these possibilities.

Materials and Methods

Animals

C57BL/6, BALB/c and DBA/2 mice were purchased from Japan SLC. *cbcl-*x_L transgenic mice in C57BL/6 background were produced in this study as described below. *bcl-2* transgenic mouse line 2 in SWR background has previously been described (Katsumata *et al*, 1992).

Construction of the Eμ-SV40-*cbcl-*x_L transgene and production of transgenic mice

Transgene construction was generated by inserting *cbcl-*x_L cDNA fragment of 1.2 kb containing the entire coding region for cBcl-x_L into the EcoRI site of the Eμ-SV40 cassette (Rosenbaum *et al*, 1989) (Figure 1). The fragment encompassing Eμ enhancer, SV40 promoter, *cbcl-*x_L cDNA and SV40 early splicing and polyadenylation signals was excised, purified, and used for microinjection. DNA at approximately 3 ng/ml was microinjected into pronuclei of eggs from inbred C57BL/6 mice and the microinjected eggs were transferred to ovarian ducts of pseudo-pregnant recipients. The methods of microinjection and embryonal transfer have previously been described (Hogan *et al*, 1986). Chromosomal integration of the transgene was initially assessed by Southern blot analysis of the genomic DNA extracted from the tails. Routinely, the transgene in progenies was detected by PCR analysis with specific primers for the *cbcl-*x_L sequences. The sequences of the primers used were: 5′ CTAGATCCCTGGATCCAGGA and 5′ TACAACAAAAGGAGCTGG-CG.

Western blot analysis

Total proteins were extracted by homogenizing mouse tissues in the extraction buffer (10 mM Tris-HCl, pH 7.4, 1% NP40, 0.1% Sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA). After electrophoresis, cBcl-x_L protein was detected with anti-cBcl-x_L polyclonal antibody raised against GST-chicken Bcl-x_L fusion protein, which is specific to cBcl-x_L and does not react to mouse Bcl-x_L (Shimizu *et al*, 1995). Thymocytes and splenocytes were prepared by pressing whole organs between sterile frosted glass slides in cold PBS, followed by lysing red blood cells with Tris-NH_4Cl solution (0.83% NH_4Cl in 17 mM Tris-HCl buffer, pH 7.2). Total protein from splenocytes and thymocytes were extracted and analyzed as above.

Immunohistochemistry

Thymus was obtained from mice perfused with fixing solution (4% paraformaldehyde in PBS) and frozen in O.C.T. compound (Miles Laboratories) at -80°C. Serial 8 to 10 μm sections were prepared and cBcl-x_L expression was examined by using avidin-biotin-peroxidase complex (ABC) technique. In brief, after incubation with 0.3% H_2O_2 in methanol to inactivate endogenous peroxidase, sections were treated with 10% normal goat serum in PBS for 20 min at room temperature and incubated with anti-cBcl-x_L antibody for 2 days at 4°C. After washing with PBS containing 0.1% Tween 20, sections were incubated with biotin-labelled goat anti-rabbit IgG antibody and then horseradish peroxidase (HRP)-conjugated avidin for 20 min each at room temperature. HRP was detected with diaminobenzidine (0.125 mg/ml in 50 mM Tris-Hcl, pH 7.6 containing 0.002% H_2O_2). Sections were counter-stained with methylgreen for histological observations.

Flow cytometric analysis

Monoclonal antibodies (MAbs) used for flow cytometric analysis were as follows: FITC-conjugated anti-murine Vβ8, anti-murine Vβ6 and PE-conjugated anti-murine Thy-1.2 and anti-murine Vβ3 (all from Pharmingen). For flow cytometry, thymocytes and splenocytes were prepared as described above. Cells were incubated with each antibody for 30 min at 4°C in 100 μl of a staining buffer (Hanks solution with 0.1% BSA and 0.1% NaN_3). After washing with the staining buffer, the cells were analyzed by a FACSort (Becton Dickinson). Apoptotic cells with subdiploid DNA were detected as described (Nicoletti *et al*, 1991). Briefly, cells were spun down by brief centrifugation and incubated in a hypotonic staining buffer (0.1% sodium citrate, 0.1% Triton X-100, 50 μg/ml of propidium (iodide) for at least 30 min at 4°C. All flow cytometric data were analyzed by Cell-Quest software (Becton Dickinson).

In vivo clonal deletion assay

In vivo clonal deletion was analyzed as previously described (Pullen *et al*, 1988). Briefly, F1 mice were obtained by intercrossing C57BL/6 *cbcl-*x_L transgenic mice with DBA/2 mice. Thymocytes and splenocytes were prepared as described above from 5 to 6 week-old F1 mice and double-stained with FITC-conjugated anti-Thy-1.2 and PE-conjugated anti-Vβ3 MAbs. The stained cells were analyzed with FACSort as described above.

In vitro deletion assay: co-culture system

In vitro deletion assay using co-culture system was carried out as described (Aiba *et al*, 1994). 1.5×10^6 thymocytes were co-cultured with mitomycin C (Wako)-treated A20.2J (gift from Dr. Katsura) antigen presenting cells (APCs) confluently covering the bottom of a 12-well plate (Costar) in the presence and absence of 10 μg/ml SEB (Sigma). The SEB concentration of 10 μg/ml was selected, based on our observations that SEB had a similar effect over a range from 5 to 20 μg/ml. After co-culturing, all cells recovered by pipetting were washed with the staining buffer and then double-stained with FITC-conjugated anti-Vβ8 and PE-conjugated anti-Thy-1.2 MAbs, or FITC-conjugated anti-Vβ6 and PE-conjugated anti-Thy-1.2 MAbs. A20.2J cells were gated out from the thymocyte due to their inability to be stained with anti-Thy 1.2 MAb.

In vitro deletion assay: fetal thymus organ culture system

From F1 embryos at day 14 gestation obtained by crossing BALB/c with *cbcl-*x_L transgenic mice (C57BL/6) or between BALB/c and *bcl-2* transgenic mice (SWR/J), thymic lobes were isolated and organ-cultured for 10 days on nucleopore filters (Costar) in RPMI 1640 medium containing 10% fetal bovine serum (FBS) as described (Takahama *et al*, 1994). Selected cultures were treated with 10 μg/ml SEB. After 18 h, cells from all cultures were obtained by mincing lobes with 27 G needles in cold PBS. The cells were double-stained with FITC-conjugated anti-Vβ8 and PE-conjugated anti-Thy-1.2 MAbs, or FITC-conjugated anti-Vβ6 and PE-conjugated anti-Thy-1.2 MAbs and analyzed with FACSort as described above.

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