

# Activation-Induced Apoptosis in T cells: Effect of Age and Caloric Restriction

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## ABSTRACT

We have previously shown that the proliferative response of T cells to antigenic or mitogenic stimulus decreased with age and that caloric resection (CR) attenuated the age-related decline in proliferation and IL-2 expression. Because activation-induced apoptosis is known to regulate cell proliferation and eliminate the high number of activated cells during an immune response, it was of interest to determine what effect aging or CR has on activation-induced apoptosis in T cells. Splenic T cells isolated from young (6-month) and old (25-month) mice fed *ad libitum* (control group) and from old (24-month) mice fed a restricted diet (40% caloric restriction) that began at 6 weeks of age. T cells were stimulated with superantigen staphylococcal enterotoxin B (SEB) or anti-CD3 antibody (primary stimulus) for 72 to 96 h, followed by restimulation with anti-CD3 (secondary stimulus). Activation-induced apoptosis was assessed by DNA fragmentation assay and the expression Fas/CD95 and Fas-ligand (Fas-L) was measured by flow cytometry. We found that the amount of DNA fragmentation was significantly ( $p<0.05$ ) increased in the stimulated and restimulated T cells from old control mice and old caloric restricted mice compared to young control mice. The increase in DNA fragmentation with age was paralleled with an increase in the proportion of the cells expressing Fas and Fas-L. However, CR had no significant effect on the age-related increase in DNA fragmentation, Fas, or Fas-L expression. We also measured the Bcl-2 and Bax protein level and found that the level of Bcl-2 decreased and Bax increased with age and that CR had no effect on the age-related changes in the level of Bcl-2 or Bax protein. These results demonstrate that aging but not CR alters activation-induced apoptosis in mice T cells. *Iran. Biomed. J. 5 (1): 1-9, 2001*

**Keywords:** T cells, Apoptosis, Aging, Caloric Restriction, Fas, Fas-L, Bax, Bcl-2

## INTRODUCTION

Stimulation of T cells with antigen or mitogen results in activation and proliferation. However, under certain conditions, especially when T cells are rested and restimulated, it can result in the induction of programmed cell death (apoptosis). This form of cell death in lymphocytes has been known as activation-induced cell death (AICD) [reviewed in 1, 2]. Apoptosis is a highly regulated event that is characterized by DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly into membrane-enclosed vesicles, apoptotic bodies [reviewed in 3, 4]. The accumulating evidence suggests that AICD serves an important immunoregulatory and homeostatic function by down-regulating the proliferating cells after repetitive stimulation [1, 2]. The mechanism is of

physiological importance because the presence of too many activated cells with different specificities might trigger excessive secondary immune responses leading to symptoms similar to autoimmune disorders and toxic shock [1, 2]. An activation-induced apoptosis model employing antigen, i.e., staphylococcus enterotoxin B (SEB) [5] or mitogens such as phytohemagglutinin (PHA) [6] concanavalin A (Con A) [7] and mitogenic antibody (anti-CD3 antibody) [8, 9] has been used to study the process of apoptosis in lymphocytes. In general, the response of mature T cells to these activating agents is characterized by T cell proliferation. However, it has been shown that some T cells undergo apoptosis when restimulated *in vitro* [2]. T cells undergoing a primary stimulation *in vitro* continue to progress through the cell cycle following restimulation but, after one or two cell divisions, many of the activated cells die by

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apoptosis despite the presence of adequate amounts of IL-2 [10, 11]. The surviving cells cannot be induced to proliferate further after a period of rest and die by apoptosis during the induction of a secondary stimulation [11, 12].

A number of molecules shown to regulate apoptosis have been identified in T cells including Fas (CD95/Apo-1) surface molecule and its specific ligand Fas-L (CD95-L). Fas belong to the tumor necrosis factor receptor/nerve growth factor receptor superfamily [reviewed in 13]. Fas protein is highly expressed on activated T cells, and it has been suggested that it may play a crucial role in activation-induced apoptosis of T cells [14-16]. Fas-L is not expressed on resting cells but increases in expression during T cell activation, and it can cross-link to Fas, leading to apoptosis [1, 2]. In addition, gene products of some members of the Bcl-2 family of protooncogenes (e.g., Bax and Bad) have been shown to promote apoptosis, whereas other members (e.g., Bcl-2 and Bcl-x<sub>L</sub>) have apoptosis-inhibiting activity [reviewed in 17, 18]. The Bcl-2 family members can bind to each other in various pairwise combinations (e.g., homodimers of Bcl-2:Bcl-2 or heterodimers like Bcl-2:Bax), and form ion-conducting channels in the mitochondria membrane [17, 18]. How Bcl-2 family members function to promote or inhibit apoptosis is uncertain. The leading theory is that the anti-apoptotic Bcl-2-like proteins inhibit caspase activation either by binding directly to a protein called Apaf-1 (apoptotic promoting activating factor-1) or by preventing the release of cytochrome c from mitochondria, or both [19, 20].

The decline in immune function with age has been extensively documented in humans and experimental animals [reviewed in 21-24]. The function of both T cells and B cells appears to change with increasing age; however, T cells seem to be more sensitive to the aging process than other cells in the immune system. Over the last decade, a variety of intervention strategies have been used in order to reverse, reduce or delay immunosenescence and the ramifications of its onset [reviewed in 25, 26]. Until now, the only robust intervention consistently shown to extend the median and the maximum life span in experimental animals and therefore, effective in retarding the process of aging, is caloric restriction (CR). There is an impressive body of evidence showing that in laboratory animals a decrease in caloric intake with maintenance of adequate levels of essential nutrients can increase longevity and postpone the

onset and lower the incidence of age-associated diseases [27-29]. Caloric restriction has been found to influence a wide variety of age-sensitive immune parameters, and overall, the immunological status of animals fed a caloric restricted diet is superior to the immunological status of the non-restricted animals [25]. Although a number of laboratories including our laboratory have investigated the effect of age and CR on immune function [reviewed in 25, 26], it is not known whether aging or CR alter the signaling event that lead to apoptosis in T cells. Because apoptosis play a major role in the development, regulation and T cells homeostasis and because the age-related defect in T cell function (proliferation and cytokine expression) is attenuated by CR, we sought to investigate the effect of age and CR on activation-induced apoptosis in T cells.

We found that the amount of DNA fragmentation in T cells from old control mice and old CR mice increased in response to the primary and secondary stimulation compared to the T cells from young control mice. The age-related increase in DNA fragmentation was paralleled with an increase in the proportion of the T cells expressing Fas and Fas-L molecules. In addition, the age-related increase in activation-induced DNA fragmentation was associated with an increase in Bax and a decrease in Bcl-2 expression. However, CR had no significant effect on the age-related increase in DNA fragmentation, Fas, Fas-L, or Bax expression. Thus, our results show that aging but not CR alters activation-induced apoptosis in T cells.

## MATERIALS AND METHODS

**Animals.** The methods of animal care and feeding were described in detail in our previous publication [30]. Briefly, at 6 weeks of age, one group (caloric restricted group) of male C57BL/6 mice (specific pathogen-free) was restricted to approximately 60% of the caloric intake of mice fed *ad libitum* (control group) throughout life. Mice were carefully examined for the presence of disease and major pathological lesions. Three groups of mice were used in these experiments: young (6-month) and old (24-month) control mice and old (24-month) caloric restricted mice. All procedures for handling the mice were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and the subcommittee for Animal Studies at Audie L. Murphy Memorial Veterans Hospital.

**Cell preparation.** Mice were decapitated, and spleens were removed aseptically. Single cell suspensions were obtained, and erythrocytes were removed using Lympholyte-M (Accurate Chemical and Scientific Corporation, Westbury, NY). B cells and macrophages were removed from T cells by a nylon wool column and the panning technique as we previously described [31]. The purity of the T cell population obtained by the panning technique is generally between 90 to 95 percent as determined by flow cytometry. T cells were resuspended in RPMI-1640 medium, which was supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). T cells were plated in a tissue culture flask and incubated in the presence or absence of stimulating agent (described below). Cells were incubated at 37°C in an atmosphere of 95% air, 5% CO<sub>2</sub>, at 90% relative humidity.

**Activation-induced apoptosis in vitro.** Activation induced apoptosis was performed as described by Radvanyi *et al.* [12]. The purified T cells (2 million cells/ml) were stimulated with mitogenic antibody (anti-CD3) or staphylococcal enterotoxin B (SEB) (10 µg/ml) plus antigen presenting cells, irradiated adherent cells (B cells and Macrophages, 1:1 ratio) after 72 to 96 h of anti-CD3 or SEB stimulation (primary stimulus). The activated cells were then harvested, re-platted in a tissue culture flask coated with anti-CD3 antibody (secondary stimulus). The anti-CD3 monoclonal antibody (PharMingen) was immobilized by coating the culture flasks with 10 µg/ml of anti-CD3 at 37°C for 2 h and then washed three times with phosphate-buffered saline (PBS). Cultured cells were supplemented with exogenous IL-2 (50 U/ml) and after 72 h of incubation, cells were harvested and the occurrence of the activation-induced apoptosis was measured.

**DNA fragmentation analysis.** Quantitation of DNA fragmentation was performed by determination of fractional solubilized DNA by diphenylamine (DPA) dye and spectrofluorimetric assay, as described by Duke & Cohen [32]. In brief, unstimulated and stimulated cells were harvested and centrifuged for 5 min at 1,000 rpm and the resulting cells pellets lysed by the addition of 0.5 ml TTE solution (10 mM Tris HCl, pH 7.4, 1 mM EDTA, 0.2% Triton X-100). The lysates were then centrifuged at 15,000 rpm for 20 min to separate intact chromatin (pellet) from soluble low molecular weight DNA (supernatant). Supernatants (containing fragmented DNA) were then transferred to a fresh tube, whereas pellets were resuspended

with 0.5 ml TTE solution. After overnight precipitation with 0.5 ml of 25% TCA (trichloroacetic acid), DNA was centrifuged for 10 min and supernatant were aspirated and discarded. The pellets were resuspended in 80 µl of 5% TCA before being placed in a heating block for 15 min at 90°C to hydrolyze DNA. Each tube was then supplemented with 160 µl of freshly prepared diphenylamine (DPA) reagents that was obtained from Sigma (150 mg DPA in a polypropylene tube, 10 ml glacial acetic acid, 150 µl concentrated sulfuric acid, 50 µl acetaldehyde (16 mg/ml), and color was allowed to develop at 37°C for 4 h. Two hundred microliters of colored solution was transferred to well of a 96-well flat-bottom ELISA plate and the optical density (OD) was determined by spectrophotometer at a wavelength of 620 nm. Percentage DNA fragmentation was calculated as the ratio of DPA fluorescence in the supernatant divided by the total fluorescence in the supernatant plus the pellet multiply by 100.

**Flow cytometric analysis of Fas and Fas-L.** The proportion of the activated T cells expressing Fas and Fas-L was assessed by flow cytometry as previously described [33]. Briefly, T cells (1-2 million) were washed and resuspended in FACS buffer (PBS with 5% FCS, and 0.1% sodium azide). Cells were stained with optimal concentration of FITC-anti-CD95/Fas antibody (PharMingen, San Diego, CA). The analysis of Fas-L expression was performed by washing the cells in FACS buffer and then incubated in permeabilized solution (0.33% saponin at 4°C) for 60 min. After washing, the cells were stained with FITC-anti-CD95-L/Fas-L antibody (PharMingen, San Diego, CA). An isotype-matched negative control was used to determine the background of fluorescence. Cells were incubated at 4°C for 30 min, washed twice with FACS buffer, and were analyzed with a flow cytometer (FACSan, Becton Dickinson). The samples were gated using forward versus 90-degree light. For each test sample, 10,000 cells were analyzed and the results were expressed as percentage of the gated cells.

**Western blotting.** The levels of Bcl-2 and Bax proteins were determined by immunoblotting as we previously described [34]. The activated cells were lysed in a protein lysis buffer (1% Nonidet P-40, 150 mM NaCl, and 20 mM Tris, pH 7.4) containing protease inhibitors (50 µg/ml aprotinin, 10 µg/ml leupeptin, 50 µg/ml pepstatin A, and 1 mM

polymethylsulfonyl fluoroide) and then incubated on ice for 30 min. The lysates were clarified by centrifugation at 4°C for 30 min at 10,000 X g, and the supernatant was assayed for protein concentration using the Bio-Rad Protein Assay Kit. Protein samples (30 µg) were boiled for 5 min with an equal volume of 2 x SDS-PAGE sample buffer and resolved on 10 or 12% SDS-PAGE. The SDS-PAGE was conducted in duplicate and one gel was stained with Coomassie blue to ensure that the same quantity of protein samples was loaded onto the gel. Protein from the gels was electroblotted onto nitrocellulose membranes and the membranes were blocked by incubating for 2 h at room temperature with 5% nonfat milk in PBS, 0.1% Tween-20. The membranes were incubated overnight with anti-Bcl-2, anti-Bax antibody (Santa Cruz, CA) or anti-actin (control) and washed three times (10 min each) with 0.1% Tween 20 in PBS. The membranes were then incubated with peroxidase-labeled IgGF (ab')<sub>2</sub> as the secondary antibody and were developed using an enhanced chemoluminescence detection system (ECL) (Amersham, Arlington Heights, IL).

**Statistic analysis.** Data were expressed as the mean ± S.E. The values for the different groups were compared with Student's *t*-test. In the present experiments, *P*<0.05 was considered statistically significant. Analysis of the data was performed using the Statistical Analysis Software GB-STAT (Dynamic Microsystem, Inc. Silver Spring, MD).

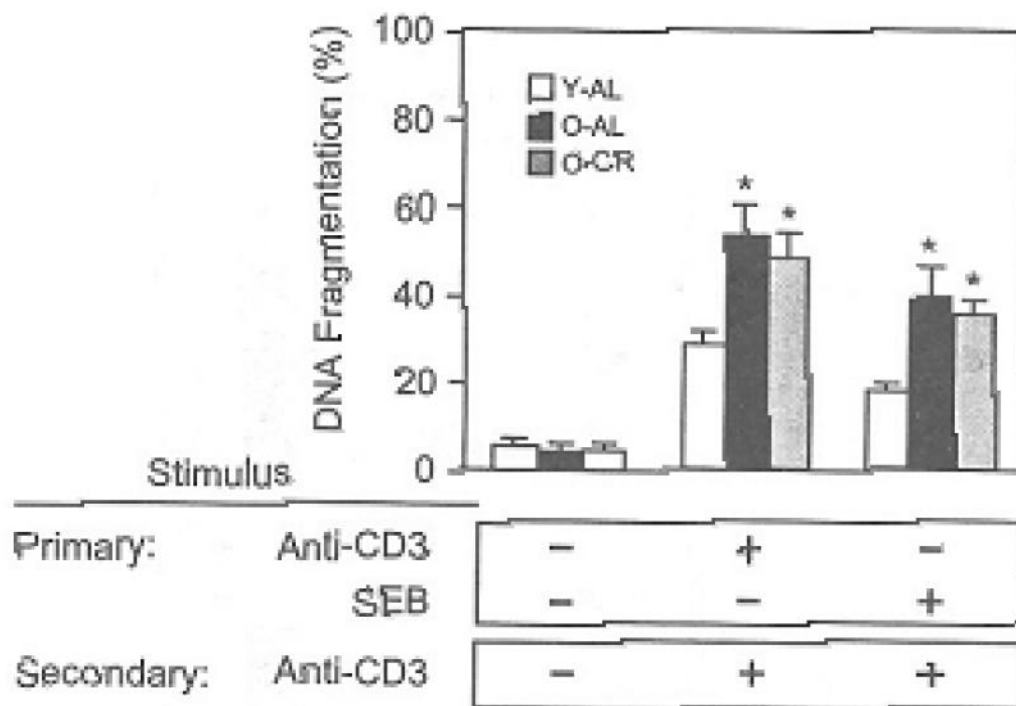
## RESULTS AND DISCUSSION

Our laboratory has previously reported that the induction of T cell proliferation and IL-2 expression decreased with age and that CR attenuated the changes [25, 26]. More recently, we found that the induction of mitogen-activated protein kinase (MAPK) activity and calcineurin phosphatase activity decreased with age [35] and that CR reduced the age-related decline in the activation of MAPK and calcineurin [36]. Research during the last several years has shown that restimulation of T cells after a short period of resting triggers the cell to undergo apoptosis. This phenomenon, which is known as "activation-induced cell death" is believed to limit cell proliferation and to eliminate the high number of activated cells during an immune response [reviewed in 1, 2]. In the

apoptotic cells, DNA is cleaved into oligonucleosomal fragments [37-39], probably through the activation of an endogenous endonuclease [38, 40]. It has been demonstrated that micrococcal nuclease-induced cleavage of DNA in isolated nuclei causes ultrastructural changes similar to those seen in apoptotic nuclei, and therefore DNA fragmentation may be the trigger for such nuclear changes in apoptosis [38].

In view of the known age-related decline in the immune system and the beneficial effect of CR on immunosenescence and given the potential important role of apoptosis in lymphocyte homeostasis, it was of interest to determine whether aging or CR alter the events that regulate activation-induced apoptosis in T cells. Our data in Figure 1 show that the DNA fragmentation increased in T cells from young and old mice after primary and secondary stimulation. However, the percentage of the fragmented DNA was higher in the stimulated and restimulated T cells from old control mice and old caloric restricted mice compared to the stimulated and restimulated T cells from young control mice. For example, 72 h after stimulation and restimulation with anti-CD3, DNA fragmentation was 56% and 48% in the activated T cells from old control mice and old caloric restricted mice, respectively, and in 28% in the activated T cells from young control mice. Although DNA fragmentation was approximately 12% lower in the activated T cells from old caloric restricted mice compared to the activated T cells from old control mice, this difference was not statistically significant. Our results demonstrate that regardless of whether cells were stimulated with anti-CD3 or SEB, the activation-induced DNA fragmentation was increased with age and that CR had no significant effect on the age-related increase in DNA fragmentation (Fig. 1).

The mechanism by which activation-induced DNA fragmentation increases with age is yet to be determined. The conventional understanding is that T cells can be induced *in vitro* to up-regulate Fas and Fas-L when cells are subjected to prolonged stimulation with mitogens such as PHA or anti-CD3 [41-46]. Fas surface protein is highly expressed on activated T cells, and it has been suggested that it may play a crucial role in activation-induced apoptosis in T cells [14-16]. Fas-L is not expressed on resting cells but increases in expression during T cell activation, and it can cross-link to Fas, leading

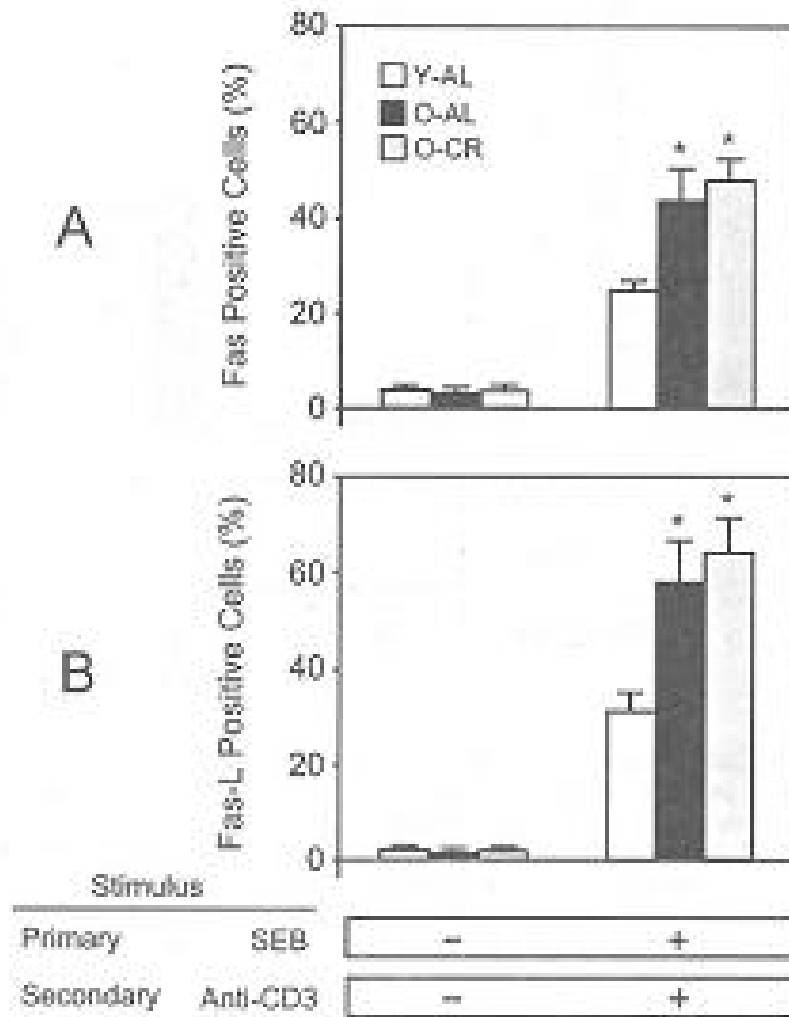


**Fig. 1.** Effect of age and caloric restriction on activation-induced DNA fragmentation in T cells from mice. Splenic T cells were isolated from young (6 month) and old (24 month) mice fed ad libitum (AL) or old mice fed a caloric restricted diet (CR). Cells were stimulated with anti-CD3 or SEB for 72 to 96 h (primary stimulus) followed by restimulation with anti-CD3 (secondary stimulus). DNA fragmentation was assessed 72 h after restimulation and the ratio of low to high molecular weight DNA was determined and expressed as percentage as described in Materials and Methods. Each point represents the mean  $\pm$  SE for data obtained from young (twelve spleens) and old (eight spleens) control (AL) mice and from old (eight spleens) CR mice. \* The values for old mice were significantly different from the value for the young mice at  $p < 0.001$ .

to apoptosis [1, 2]. In addition, an increase in Bax protein has been associated with promotion of apoptosis and converse effects occur when the level of Bcl-2 protein increases [reviewed in 17, 18]. To establish that the increase in DNA fragmentation that we have observed with age was associated with an increase in the proportion of the activated T cells expressing Fas or Fas-L, we measured the percentage of Fas<sup>+</sup> and Fas-L<sup>+</sup> cells in the activated T cells using flow cytometry. Figure 2 shows that the proportion of T cells expressing Fas surface molecule increased after primary (SEB) and secondary (anti-CD3) stimulation. However, the percentage of Fas<sup>+</sup> T cells was higher in the activated T cells from old control mice and old caloric restricted mice compared to the activated T cells from young control mice. For example, Fas surface protein was expressed in 43% and 48% of the activated T cells isolated from old control mice and old caloric restricted mice, respectively, and in 22% of the activated T cells from young control mice. Similarly, the expression of Fas-L was 90% to

100% higher in the activated T cells from old control mice and old caloric restricted mice than activated T cells from young control mice (Fig. 2). The proportion of Fas<sup>+</sup> and Fas-L<sup>+</sup> cells was approximately 10% to 12% higher in the activated T cells from old caloric restricted mice compared to old control mice, but this difference was not significant.

Because Bcl-2 and Bax proteins have been implicated in the regulation of apoptosis [reviewed in 17, 18], we also measured the levels of Bcl-2 and Bax proteins in the stimulated and restimulated T cells by Western blot analysis using a specific antibody that recognized each protein or antibody against actin as a control. Figure 3 shows the Bcl-2 protein level was 59% and 47% lower for the activated T cells from old control mice and old caloric restricted mice, respectively, compared to the activated T cells from control young mice. In contrast, Bax protein levels were 90% and 120% higher for the activated T cells from old control mice and old caloric restricted mice, respectively,

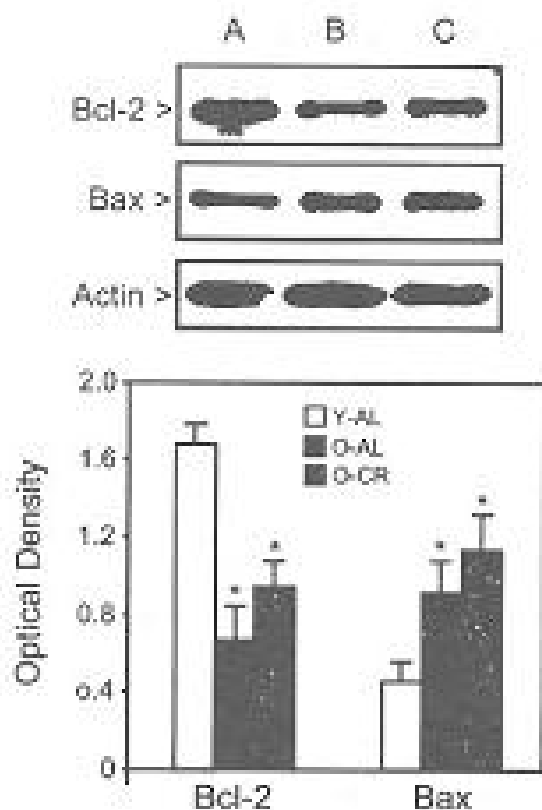


**Fig. 2.** Effect of age and caloric restriction on activation-induced Fas and Fas-L expression. Splenic T cells from young and old control mice and from old CR mice was incubated with SEB for 96 h, followed by restimulation with anti-CD3 for 72 h. Cells were stained with anti-Fas or anti-Fas-L antibody and the percent Fas positive cells (A) and Fas-L positive cells (B) were assessed by flow cytometry as described in the Methods. Each point represents the mean  $\pm$  SE for data obtained from young (six spleens) and old (four spleens) control (AL) mice and from old (four spleens) CR mice. \* The values for old mice were significantly different from the value for the young mice at  $p < 0.05$ .

than the activated T cells from young control mice. Although the Bcl-2 and Bax protein level was slightly higher in the caloric restricted old mice compared to the old control mice, this difference was not statistically significant. Thus, our results for the first time show that activation-induced apoptosis as assessed by DNA fragmentation, Fas, Fas-L, Bcl-2, and Bax expression alter with age and that CR had no significant effect on the age-related changes in the apoptotic parameters that were measured.

It has been demonstrated that the proportion of memory T cells increases and the proportion of the naive/virgin T cells decreases with age [24, 47].

Therefore, one possible explanation for the increase in activation-induced apoptosis in T cells from old mice that we have observed in this study may be due to differential sensitivity of T cell subpopulations (i.e., naive/virgin and memory) to apoptosis. It is possible that the naive T cells are more sensitive to activation-induced apoptosis than memory T cells. This view is supported by a study, which showed that the accumulation of memory T cells in elderly human is not due to an increase in generation of these cells, but rather due to a selective apoptosis of naive cells [48]. A study in humans showed that the expression of Fas increased



**Fig. 3.** Effect of age and caloric restriction on the induction of Bcl-2 and Bax expression. Splenic T cells from young (A) and old (B) mice and from old (C) CR mice were incubated with SEB for 96 h, followed by restimulation with anti-CD3 for 72 h. Bcl-2 and Bax protein levels were determined by Western blots as described in the Methods. The blots were probed with anti-actin antibody as a control. The autoradiograph is shown and the bands corresponding to Bcl-2 and Bax were quantified by densitometry, and the data are presented in the graph. Each point represents the mean  $\pm$  SD for data obtained from three experiments and each experiment was pooled from two mice. \* The values for old mice were significantly different from the value for the young mice at  $p < 0.001$ .

with age and that Fas expression was more pronounced in memory (CD45RO<sup>+</sup>) T cells than the naive (CD45RO<sup>-</sup>) T cell populations [49]. Naive T cells have been shown to be susceptible to Fas-mediated apoptosis when subjected to strong TCR ligation [50]. Similarly, a recent study shows naive T cells die as a consequence of Fas ligation in the presence of anti-CD3 stimulation, whereas memory T cells subjected to same stimulation resulted in proliferation by Fas ligation [51]. This study

concluded that naive and memory T cells display dichotomous responses to Fas ligation.

Our results corroborate with recent reports that show apoptosis increases with age in rat hepatocytes [52, 53]. However, it does not confirm the reported study that show the inducibility of apoptosis by dexamethosone decreases with age and that CR significantly reversed the age-related decline in apoptosis [54]. The difference in this study and our finding may reflect differences in the experimental design, the assay system, cell population analyzed and/or strain of rodents studied. For example, in this study [54], T cells were stimulated once with anti-CD3 for only 24 h. Apoptosis was assessed by measuring the surface expression of phosphatidylserine by staining with annexin V and determining the expression of annexin V as an indicator of apoptosis. These potentially confounding factors could contribute to the discrepancy between this study and our results. While it appears that improved immune function may contribute fundamentally to mechanism by which CR enhances apoptosis and reduces tumor [55], the currently available data are insufficient to determine the extent of this contribution.

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