Calorie restriction increases Fas/Fas-ligand expression and apoptosis in murine splenic lymphocytes

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Abstract One-month-old male ICR mice were fed a nutritionally adequate, semipurified diet, either ad libitum (AL) or calorie restricted (CR) (40% less food) for 6 months and were killed to obtain spleens. Flow cytometric analysis revealed increased proportions of both $CD4^+$ and $CD8^+$ T cells in CR-fed mice compared to AL-fed mice. The T cell subsets of CR-fed mice were also found to have higher levels of plasma membrane Fas receptor expression. Similarly, Fas-ligand (Fas-L) expression was higher in anti-CD3-stimulated $CD4^+$ and $CD8^+$ T cells. CR-fed mice also had increased numbers of annexin V-positive $CD4^+$ and $CD8^+$ T cells in stimulated splenic lymphocytes suggesting an increased potential for apoptosis. Fas and Fas-L gene expression in splenic lymphocytes, which correlated closely with the observed increased rate of apoptosis, was significantly increased in CR-fed mice compared to AL-fed mice. In conclusion, these results indicate that CR increases the expression of Fas and Fas-L which may contribute to the known beneficial effects of CR such as prolongation of life span by activating chronic physiologically mediated apoptosis.

Key words: Calorie restriction; Fas (CD95); Fas-ligand (CD95L); Apoptosis; Splenic lymphocyte subset

1. Introduction

One third of all cancer-related deaths, world wide, are closely linked to dietary factors [1]. Particularly, excess caloric intake appears to be the most important causative factor in promoting obesity and various malignancies. In several rodent models, overall dietary or caloric restriction alone is found to decrease tumor incidence [2–4]. Moderate calorie restriction (CR) (40% less food intake) is well established in the prevention and/or delay of onset of age-associated, life-shortening diseases (e.g. breast cancer and kidney disease) in rodents [5–7]. In autoimmune-susceptible mice, CR is found to delay the loss of immune function and prolong the life span [6,7]. CR was also found to lower the incidence of breast and liver tumors by increasing apoptosis and by increasing antioxidant enzyme mRNA thereby decreasing the proliferative rate of tumor cells [8–10].

Apoptosis (programmed cell death) plays a central role in embryogenesis, morphogenesis, and immune cell regulation and is likely to act as a defense mechanism against infectious diseases and also during aging. Apart from its role in normal cell regulation, apoptosis is observed in a variety of pathological processes such as cancer, neurodegenerative disorders, and autoimmune diseases (e.g. arthritis and systemic lupus erythematosus) [11–13].

Intracellular pathways leading to apoptosis are as complex as those involved in cellular growth and differentiation [14]. Impaired growth signaling pathways, including apoptotic signal transduction, may lead to the onset of a variety of diseases [14]. Apoptosis is closely regulated by a number of gene products that either promote cell death or extend cell survival [15,16].

Fas/APO-1 (CD95) is a cell surface receptor of the tumor necrosis factor (TNF) receptor superfamily that mediates T and B lymphocyte apoptosis [17]. Fas-ligand (Fas-L), expressed predominantly on activated T lymphocytes [18], mediates cell death by cross-linking to the Fas receptor [18,19]. Earlier studies, including our own, showed that CR modulates lymphocyte subsets and increases apoptosis [20,21]. Involvement of Fas and Fas-L in both peripheral lymphoid cell death and lymphoid organ development is well-established [22]. Activation-induced apoptosis is a primary mechanism for the down regulation of an immune response leading to immune homeostasis and defective T cell specificity [23].

The present investigation was aimed at determining the effect of CR on induction and expression of Fas and Fas-L, and the rate of apoptosis in splenic lymphocytes of ICR mice.

2. Materials and methods

2.1. Materials

Brefeldin A, ionomycin, and phytohemagglutinin (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone, annexin V, propidium iodide (PI) and all FITC and PE labeled antibodies and antibody isotype controls were purchased from Pharmingen (San Diego, CA, USA). All other chemicals (analytical grade) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Outbred, weight-matched, male ICR mice (albino, weanling mice obtained from Taconic, NY, USA) at 4 weeks of age were grouped and housed, five mice per cage in a temperature-controlled room at 24°C and maintained on a 12:12 h dark-light cycle, in the laboratory animal care facility at the University of Texas Health Science Center at San Antonio. Mice were fed a nutritionally adequate AIN^{76} formula semi-purified diet containing 5% (w/w) corn oil (CO), either ad libitum (AL) or 40% CR as described earlier [24]. Nutrient intake was sufficient to prevent deficiency. The composition of the diet was 20% casein, 50% dextrose, 15% starch, 5% cellulose, 5% corn oil, 3.5% AIN salt mixture, 1% AIN vitamin mixture, 0.3% DL-methionine and 0.2% choline chloride

After 28 weeks of feeding, mice were fasted overnight and given a mild anesthesia. Peripheral blood was then collected by retro-orbital bleeding, and mice were killed by cervical dislocation. Spleens were

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Abbreviations: AL, ad libitum; BSA, bovine serum albumin; CO, corn oil; CR, calorie restriction; Fas-L, Fas-ligand; ME, mercaptoethanol; PI, propidium iodide; PHA, phytohemagglutinin; TBA, thiobarbituric acid; TNF, tumor necrosis factor

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aseptically removed and placed in RPM1 1640 complete medium (containing 10% heat inactivated FCS, 100 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 μM 2-mercaptoethanol (ME)). Serum was separated by centrifuging the whole blood at $1000\times g$ for 15 min and analyzed immediately. All procedures were approved by the Institutional Animal Care and Use Committee.

2.3. Splenocyte preparation

Single cell suspensions were prepared by disrupting the spleen between frosted glass slides in RPMI 1640 medium with 2% heat-inactivated fetal calf serum. After a 5 min centrifugation at $100\times g$ to separate cells from debris, the cells were washed twice in RPMI medium [25]. Spleen lymphocytes were purified by layering over lympholyte (Cedarlane Labs. Lit., Hornby, Ont., Canada), centrifuging at 1000 rpm for 15 min at 22°C, then washing twice in RPMI 1640 complete medium. Cells were counted and viability was determined by trypan blue exclusion.

2.4. Quantification of apoptosis and necrosis

Lymphocytes, 1×10^6, were incubated for 24 h in RPMI complete medium with or without 2 μg anti-CD3 monoclonal antibody (mAb) in a final volume of 1 ml in a 5% CO_2-humidified incubator at 37° C. After incubation, cells were washed twice with binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), resuspended in 200 μl binding buffer in the presence of 0.2 μg FITC-annexin V and 2 μg PI then incubated in the dark for 10 min [26]. Annexin V and PI staining, as indicators of apoptosis and necrosis respectively, were measured with the FACScan (Becton Dickinson) using the Cell Quest software as described earlier [27].

2.5. Flow cytometric analysis of Fas, Fas-L and annexin V staining of splenocyte subsets

Cells were washed with isotonic NaCl/phosphate buffer containing 0.5% bovine serum albumin (BSA) and 0.2% NaN_3 (wash buffer). Cells (1×10^6) were incubated with 0.1 μg of Fc block at room temperature for 10 min. After two washes in wash buffer, 1×10^5 cells in 200 μl wash buffer were incubated at 4°C for 30 min with 2 μl of one of the following pairs of monoclonal antibodies: anti-CD4-PE/anti-Fas-FITC or anti-CD8-PE/anti-Fas-FITC. An isotype control for anti-Fas-FITC was used in all experiments.

Because Fas-L is expressed on activated T cells, splenocytes (1×0^6 cells) were activated in the presence of soluble anti-CD3 mAb (2 μg/ml) for 24 h and after washing, the cells were further stimulated with brefeldin A, PMA and ionomycin (2 μg, 10 ng and 1 μg/ml respectively) for 4 h in a humidified atmosphere at 37°C with 5% CO_2. Cells were blocked, then cell aliquots incubated for 30 min with anti-CD4 or -CD8 FITC-labeled mAb as described above. Cells were then fixed with 200 μl of 1% paraformaldehyde in wash buffer for 15 min with continuous shaking at room temperature. Lymphocytes were washed with wash buffer, and then permeabilized by suspending in 200 μl of 0.33% saponin at 4°C for 45 min. Permeabilized cells were washed and stained with anti Fas-L-PE and isotype anti-Fas-PE in 200 μl wash buffer at 4°C for 45 min.

2.6. Annexin V staining of splenocyte subsets

Cells incubated with medium alone or with anti-CD3 were blocked with Fc receptor and cell aliquots incubated for 30 min with anti-CD4- or CD8-PE-labeled mAb as described above. Cells were washed twice with binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), then incubated with 0.2 μg of annexin V-FITC for 15 min in the dark. Cellular fluorescence was analyzed by flow cytometry (Becton Dickinson FACScan flow cytometer) using the Cell Quest program. (All antibodies were purchased from Pharmingen, San Diego, CA, USA.)

2.7. Reverse transcription (RT)-PCR of Fas and Fas-L mRNA

Cellular RNA was isolated from 3×10^6 cells using Trizole reagent (Gibco BRL, Life Technologies Grand Island, NY, USA) as described by the manufacturer. Reverse transcription of 1 μg RNA was performed using oligo-dT, 10 mM dNTPs, 20 units RNase inhibitor and reverse transcriptase (Gibco BRL, Life Technologies) in a total volume of 20 μl. PCR was performed in a mixture containing 3 μl cDNA and 1 μM primers of Fas or Fas-L (CLP continental Lab products Cat. Nos. 5245.M and 5246.M). Amplification was performed using Taq-polymerase (Promega, Madison, WI, USA) with one initial denaturation cycle for 2 min at 94°C, 2 min at 60°C; 25 cycles for 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C; and a final extension phase consisting of one cycle of 2 min at 72°C. The GAPDH (3′ primer TCC ACC ACC CTG TTG CTG TA and 5′ primer ACC ACA GTC CAT GCC ATC AC) housekeeping gene was used as a control. PCR products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide and visualized by UV light illumination. Band density was measured using an Alpha Imager 2000 (Alpha Innotec Corporation).

2.8. Statistical analysis

The data shown are the mean ± S.E.M. of five individual values. Data were statistically analyzed using the Student's *t*-test and $P<0.05$ was considered significant.

3. Results and discussion

In the present study, mice on the CR diet gained significantly less (51%) body weight (23 ± 0.5 vs. 47.2 ± 0.44), compared to mice on the AL diet. Similar results without any malnourishment were also observed in earlier studies [21,28].

The influence of CR on lymphocyte subsets is summarized in Fig. 1. Similar to previous reports [20,21], the present study also showed that CR mice maintain a significantly higher percentage of $CD4^+$ and $CD8^+$ splenocytes (15 and 35% respectively), compared to AL mice. Further, CR also significantly decreased $CD19^+$ splenocytes (22%). Decreased B lymphocytes in CR can contribute to lowering the incidence of B cell lymphoma in autoimmune diseases [29].

Interestingly, CR significantly modified the phenotypic expression of Fas and Fas-L in activated T lymphocytes (Figs. 2 and 3). Two-color flow cytometric analysis showed that the overall percentages of Fas^+ cells in $CD4^+$ and $CD8^+$ lymphocytes of CR mice were increased by 13 and 24% respectively, compared to that of AL mice. Similarly, the percentages of $Fas\text{-}L^+$ cells in $CD4^+$ and $CD8^+$ lymphocytes of CR mice were increased by 38.8 and 39.6% respectively, compared to AL-fed mice.

The main function of Fas antigen is to trigger the induction of apoptosis [30]. The Fas receptor was first described as a molecule expressed on the surface of certain cell lines, which could mediate apoptosis when triggered by a specific monoclonal antibody [31]. The Fas system plays an important role in modulating B and T lymphocyte development and their function [32]. Further, binding of Fas-L to Fas antigen results in intracellular signals leading to apoptosis [30]. Fas-L is one

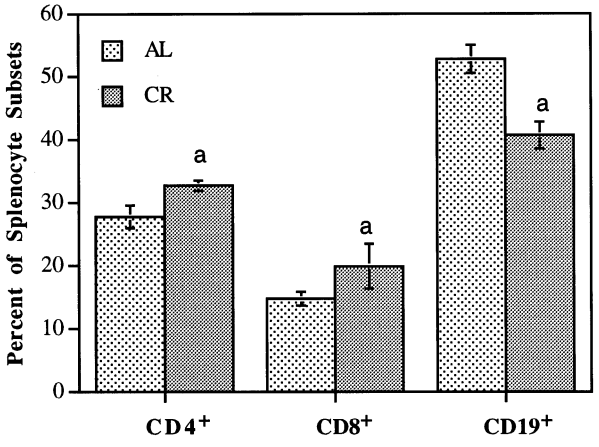

Fig. 1. Effect of CR and AL on splenocyte subsets. Values are mean ± S.E.M. of five individual values. [a]Statistically significant at $P<0.05$.

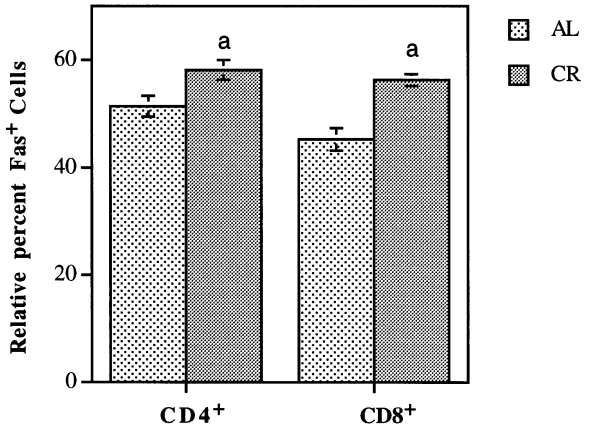

Fig. 2. Effect of CR and AL on phenotype expression of Fas on splenocyte subsets. Values are mean ± S.E.M. of five individual values. [a]Statistically significant at $P < 0.05$.

of the major effectors of $CD8^+$ cytotoxic lymphocytes and is involved in the establishment of peripheral tolerance [33] and the activation induced cell death of lymphocytes [19,34]. It is well established that apoptosis is a distinct form of cell death that is essential to the regulation of the immune system. In this study, apoptosis was analyzed by flow cytometry, using both annexin V and PI staining. Because phosphatidylserine is exclusively located in the cell membrane leaflets that face the cytosol in live cells, the surface expression of phosphatidylserine is a feature of apoptosis, and it occurs before the loss of membrane integrity [35]. It is also known that early apoptotic cells bind annexin V, a Ca^{2+}-dependent phospholipid binding protein with high affinity for phosphatidylserine, but exclude PI and can therefore be specifically detected and quantified by FACS analysis [36]. Annexin V staining for apoptosis was significantly increased in both $CD4^+$ and $CD8^+$ cells of CR mice incubated with anti-CD3 (67 and 40%, respectively), or medium alone (49.9 and 31.5%, respectively) compared to AL-fed mice (Fig. 4). Spontaneous, as well as anti-CD3 mAb-induced apoptosis in total lymphocytes was also increased, by 39.3 and 60.7% respectively, in CR mice compared to AL-fed mice (Fig. 5). However, CR did not change the necrotic cell population, as measured by double staining with annexin V and PI, which may indicate advanced apoptotic cells rather than necrotic cells (Fig. 6). Previous studies also showed a significantly increased apoptosis in CR-fed autoimmune-prone and -resistant strains of mice [21,37].

Increased Fas expression on $CD4^+$ and $CD8^+$ cells in CR-fed mice is most likely physiologically significant. In this study we have demonstrated that increased expression of Fas and Fas-L on T lymphocytes is directly correlated with increased apoptosis in $CD4^+$ and $CD8^+$ cells from CR mouse spleens. Defects in the Fas/Fas-L pathway have been shown to result in autoimmune disease [23]. Defective antigen-mediated cell death can contribute to an increased number of autoreactive cells [38]. Loss of apoptotic function due to mutations in murine Fas-L (gld) or Fas (lpr) or human Fas or Fas-L leads to lymphoproliferation, lymphadenopathy and autoimmune disease [39]. The physiological regulation of programmed cell death is essential for removal of potentially autoreactive lymphocytes and prevention of an autoimmune response. It is now widely accepted that apoptosis plays an important role in the selection of immature thymocytes to maintain functionally active peripheral T lymphocytes [40]. In addition, B lymphocytes are thought to die by apoptosis during their development in the bone marrow [41]. Results presented here suggest that CR enhances the sensitivity of splenocytes to activation-

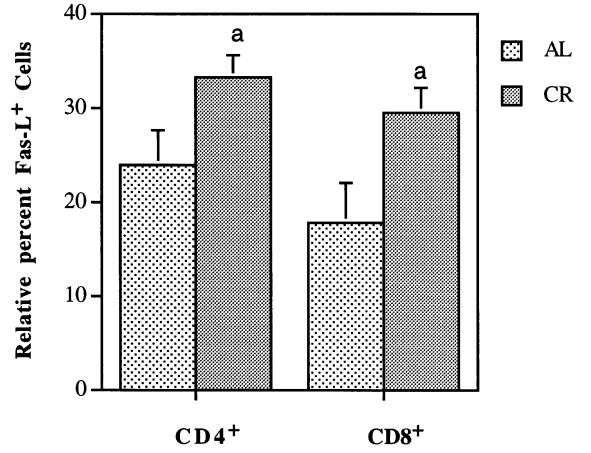

Fig. 3. Effect of CR and AL on phenotype expression of Fas-L on splenocyte subsets. Values are mean ± S.E.M. of five individual values. [a]Statistically significant at $P < 0.05$.

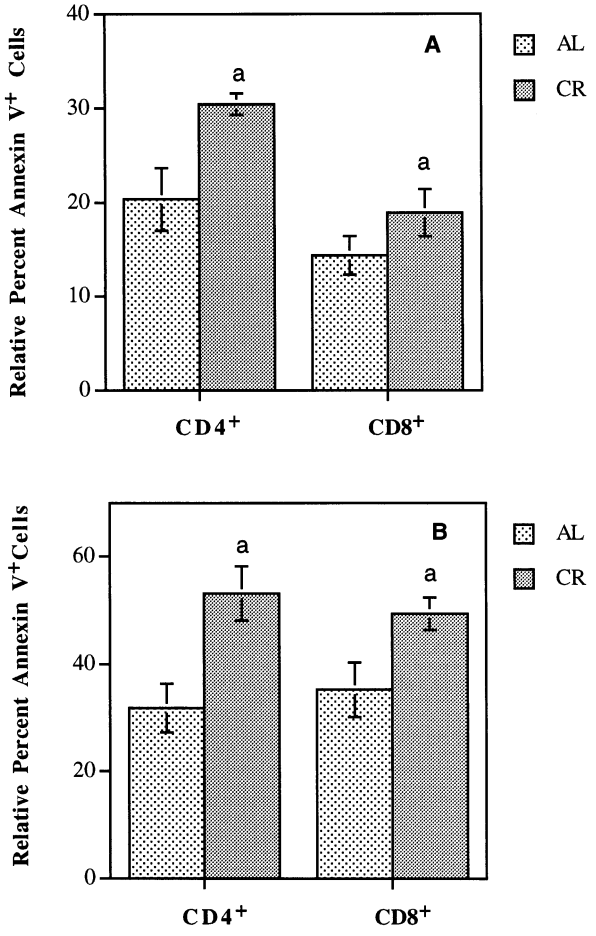

Fig. 4. Effect of CR and AL on annexin V binding to splenocyte subsets. Splenocytes were incubated for 24 h in (A) medium alone or (B) medium with anti-CD3, as described in Section 2. Values are mean ± S.E.M. of five individual values. [a]Statistically significant at $P < 0.05$.

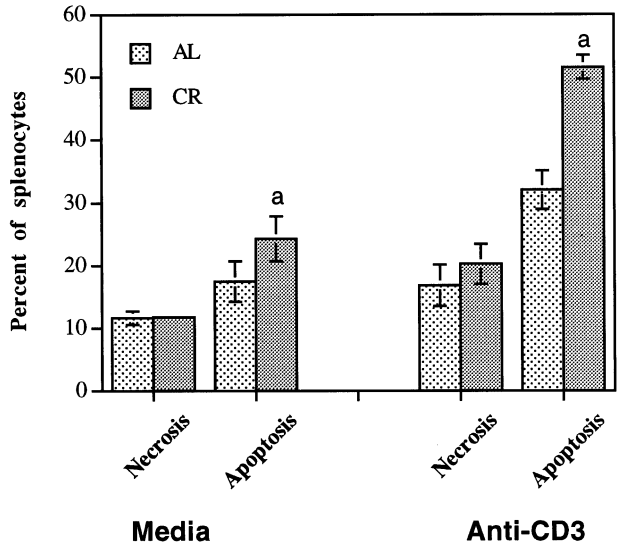

Fig. 5. Effect of CR and AL on necrosis and apoptosis in splenocytes. Splenocytes were incubated for 24 h in medium alone or medium with anti-CD3, as described in Section 2. Values are mean ± S.E.M. of five individual values. [a]Statistically significant at $P < 0.05$.

induced cell death leading to elimination of hyperreactive lymphocytes. Further, there is increasing evidence that alteration in cancer cell sensitivity to Fas-mediated apoptosis is a key factor in controlling tumor progression [42]. Perturbations in the Fas/Fas-L cell death pathway also appear to be important in determining the viability of transformed cells [43].

The role of CR in increasing apoptosis in other organs, such as liver, has also been recently described [10,44]. Further, CR was shown to lower the incidence of hepatocarcinoma and decrease the proliferation of murine hepatocytes. Decreased carcinogenesis and tumor formation in CR-fed mice has been suggested to be due to increased apoptosis [10]. This increase in the intrinsic rate of apoptosis may contribute, in part, to the decreased tumor incidence and increased life span observed in CR rodents [37].

Dietary alterations are known to have a marked influence on the expression of several genes. Diet or hormones, or their metabolites, may cause coordinated changes in protein synthesis and the abundance of mRNA, indicating that cellular control is at the molecular level [45,46]. We analyzed the expression of Fas- and Fas-L-bearing cellular mRNA by RT-PCR in stimulated and unstimulated freshly isolated splenocytes (Fig. 7A) and found that Fas and Fas-L were significantly increased in CR-fed mice. Analysis of the Fas and Fas-L/GAPDH mRNA product ratios indicates that Fas and Fas-L expression is significantly increased by 12.5 and 32% respectively, in the splenocytes of CR mice, compared to AL mice (Fig. 7B).

Earlier findings from our laboratory showed decreased mRNA expression of proinflammatory cytokines (interleukin-6, tumor nerosis factor α), and increased expression of the anti-inflammatory cytokine, tumor growth factor β in CR mouse spleen cells [7], indicating that CR modulates the expression of various cytokines and growth factors. We have also reported that CR significantly inhibited the development of mammary tumors and altered the expression of both cytokines and oncogenes, particularly elevation of p53, which was noted in tumor tissues of CR-fed mice. We hypothesized that elevation of p53 by CR may contribute to increased apoptosis of tumor cells. Indeed, 80% of AL-fed mice developed breast tumors, whereas only 30% of CR-fed mice had visible breast tumors. Furthermore, in the same breast tumor tissues, CR increased free radical scavenging enzyme expression [4].

In conclusion, our results indicate that CR increases the expression of apoptotic mediators such as Fas and Fas-L in splenocytes which may facilitate increased cell death and

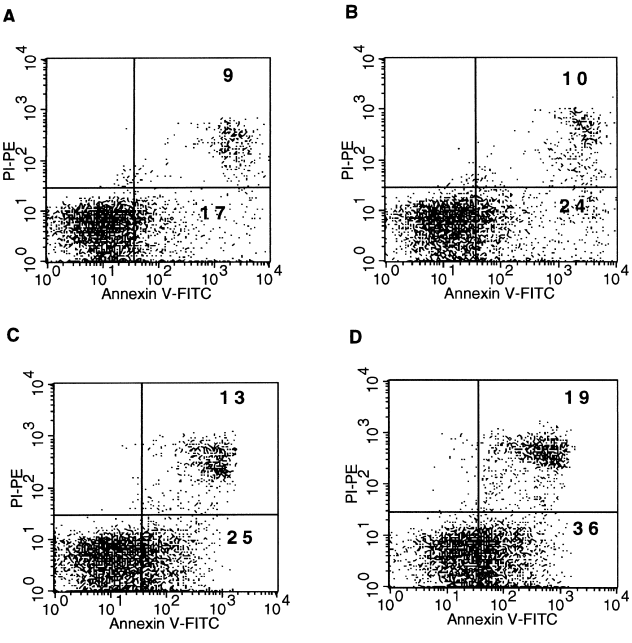

Fig. 6. A representative contour plot (out of five) showing the effect of CR on apoptosis and necrosis. Annexin V^+ (lower right quadrant) indicates apoptotic and annexin V^+ and PI^+ (upper right quadrant) indicates necrotic (advanced apoptosis) populations. Cells were incubated either with medium (A and B) or with medium with anti-CD3 (C and D). A and C: AL; B and D: CR.

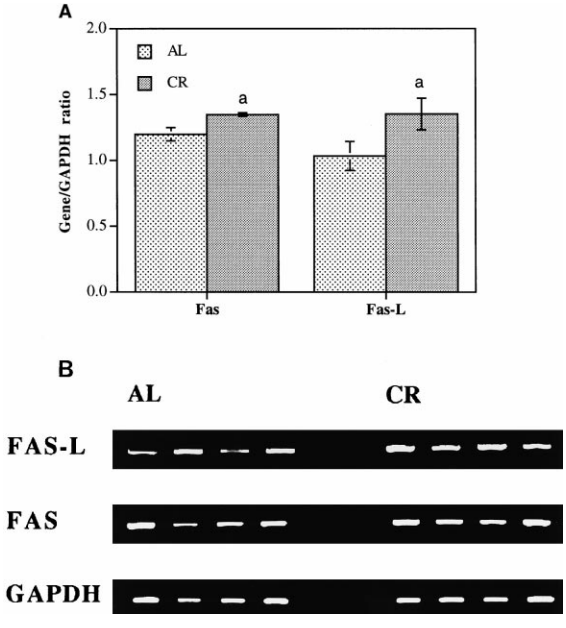

Fig. 7. A: Ratio of Fas and Fas-L/GAPDH mRNA in spleens of AL and CR mice. Values are mean ± S.E.M. of four individual values. [a]Statistically significant at $P < 0.05$. B: Representative gel of Fas, Fas-L and GAPDH mRNA in spleen of AL and CR mice.

thereby prevent accumulation of autoreactive immune cells. It thus appears that by enhancing the cell death potential, CR is likely to play a major role in controlling cancer and autoimmune diseases and thereby facilitate the prolongation of the life span [47,48].

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