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Caloric restriction reduces IgA levels and modifies cytokine mRNA expression in mouse small intestine

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Abstract

The aim of this study was to determine the effect of caloric restriction (CR) in mouse small intestine on the production and secretion of immunoglobulin (Ig) A, the population of lymphocytes in the lamina propria, and the expression of cytokines that mediate and regulate innate and adaptive immunity. One group of young Balb/c mice was fed *ad libitum*, while the CR group was fed *ad libitum* and fasted on alternate days. When mice were six months old, IgA levels in the proximal small intestine were quantified by enzyme-linked immunosorbent assay, while the number of IgA containing cells, $CD4^+$ T cells and $CD8^+$ T cells in the duodenal mucosa was determined by immunohistochemistry. Furthermore, the expression of several intestinal cytokines, the genes for α-chain IgA, and the polymeric Ig receptor (pIgR) were analyzed by real-time polymerase chain reaction. CR decreased the levels of IgA in the intestine, apparently a consequence of a reduced number of IgA^+ cells in the lamina propria that decrease the production and secretion of this Ig, and a reduced secretion of S-IgA into the bile, which in turn discharges into the proximal intestine. Contrarily, CR increased the expression of genes for α-chain IgA, and the pIgR, indicating that transport of IgA was not a key factor in the decrease of this Ig. Additionally, CR modified the expression of genes for tumor necrosis factor-α, interferon-γ, tumor growth factor-β, interleukin (IL)-2 and IL-10, all of which regulate the synthesis of IgA and pIgR, the inflammatory response, and the immune response in the intestine.

Keywords: Caloric restriction; Secretory IgA; Polymeric immunoglobulin receptor; Cytokines; Mucosal immune response

1. Introduction

Moderate caloric restriction (CR) has several effects on immune responses in both rodents and humans. In general, CR has beneficial effects on immune functions that depend on T lymphocytes, thus preventing many types of immune-mediated diseases such as autoimmunity, cancer and ageing [1,2]. In contrast, long-term CR may increase the susceptibility of the host to intact pathogens [3,4].

Some specific effects of moderate CR are a lower proliferation of antigen-specific T cells, a decreased production of cytokines, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-2, a reduced autoantibody production, and an increased natural killer cell activity. Additionally, CR preserves the population of "naïve" T cells, slows the emergence of memory T cells [5], and increases the number of cytotoxic T cells, the latter increasing resistance to viral infection [6,7]. On the contrary, CR inhibits some functions of monocytes/macrophages, such as the production of oxygen free radicals, production of cytokines (e.g., TNF-α), phagocytosis and antigen presentation [8,9], with the consequence of possibly increasing susceptibility to infections [4]. Moreover, CR increases production of prostaglandin E_2, which has inhibitory activity on macrophage activity [1].

The information about the effects of CR on the mucosal immune system is sparse. One study showed that CR reduces the production of proinflammatory cytokines and the expression of the polymeric immunoglobulin (Ig) receptor (pIgR) [10]. Another reports that a moderate energy restriction does not affect the secretion of IgA, lactoferrin or lysozyme in human milk [11].

Despite the available information, there is not any general hypothesis to explain the beneficial effects of CR on the systemic immune system [12]. Therefore, more studies are needed to determine the mechanisms by which CR prevents many different diseases, especially those related to mucosal immunity and IgA levels. IgA is a particularly important factor, as secretory immunoglobulin A (S-IgA) is the most abundant and principal intestinal immunoglobulin of humans and most other mammals.

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There are two principal mechanisms by which intestinal IgA levels are determined in humans. Firstly, intestinal IgA is produced and secreted by IgA^+ cells of the lamina propria. Secondly, IgA is secreted into the intestinal lumen by the cooperation of local plasma cells with epithelial cells. The polymeric IgA (pIgA) secreted by plasma cells diffuses through the stroma and binds to the pIgR on the basolateral surface of the epithelial cells to form the pIgA–pIgR complex, which in turn is translocated to the apical surface of the epithelial cell where it is cleaved and secreted into lumen as S-IgA [13–15]. By binding to antigens, S-IgA protects intestinal mucosal surfaces against colonization and invasion by pathogens [13–18].

In the present study an assessment was made of the effect of CR on intestinal IgA levels, lymphocyte populations (especially IgA^+, B and T cells), and gene expression encoding for cytokines, α-chain of IgA and the pIgR. To the best of our knowledge, there are no previous reports of such studies. Further studies are needed that evaluate the effects of CR on intestinal immunity, and such contributions could justify the use of CR to prevent common diseases in human populations [1,19].

2. Materials and methods

2.1. Animals, experimental groups and CR protocol

Nine week old male Balb/c mice were obtained from our Animal Breeding Unit (Escuela Superior de Medicina, Instituto Politecnico Nacional) and were randomly placed in two groups, each one of $n=8$. Each animal was separately housed in a plastic cage, in a room with a 12-h light/dark cycle (lights at 8 a.m.) during 18 weeks. Animals were treated according to a protocol approved by the Ethics and Institutional Animal Care and Use Committees. From the 10th to the 27th week of life, the *ad libitum*-fed (AL) group was fed with the NIH-31 diet *ad libitum*, while the CR group was alternately fed with the NIH-31 diet *ad libitum* and fasted. Food was always removed and replaced at 1 p.m. All groups were anaesthetized with diethyl-ether and sacrificed by cervical dislocation.

2.2. Collection of biological samples

Immediately after the mice were sacrificed, the proximal small intestine was removed and 5 ml of phosphate-buffered saline (PBS) containing 0.02% sodium azide was passed through it in order to collect the intestinal fluid. The first intestinal wash removed 85–90% of total IgA, the variability from sample to sample being less than a 5%. The washout material was centrifuged at 10,000×*g* for 20 min at 4°C. After the supernatant was harvested, it was stored at −70°C to await analysis. Total IgA was measured by enzyme-linked immunosorbent assay (ELISA).

2.3. Immunohistochemistry and measurement of IgA^+ cells

One-centimeter sections of duodenum were frozen and maintained in isopentane at −70°C until they were cut with a cryotome. Sections of 7 μm were fixed with acetone for 20 min, then hydrated with PBS. The endogenous peroxidase was blocked by incubation for 10 min. with 0.3% of H_2O_2 and 0.1% of NaN_3 in PBS. After washing, the samples were incubated for 30 min. with 5% bovine serum. Sections were then washed in 0.05% Tween-20 in PBS (PBS-T) and later incubated for 1 h in a humidified chamber at room temperature (RT) with goat anti-mouse IgA peroxidase conjugate (US Biological, Swampscott, MA, USA, 11890-14G). The procedure for control groups included omission of the primary antibody and replacement of the specific primary antibody with normal goat serum. After washing, the activity of peroxidase was revealed with H_2O_2 diaminobenzidine (Pierce, USA, 34002). Samples were counterstained with Harris' haematoxylin, then dehydrated and covered with synthetic resin (Merck, Naucalpan de Juárez, Estado de México, México). Negative controls were processed in the same way, but incubated only with PBS. Plasma cells containing IgA (IgA^+ cells) were quantified in random fields at a magnification factor of 400 by using the image analysis program Imagen-Pro Plus 5.1. Fields were adjusted to give the number of cells per 0.05 mm^2 of tissue. Tissue sections were analyzed by two independent observers.

2.4. Immunohistochemistry and measurement of intestinal lymphocytes

Intraepithelial and lamina propria (LP) lymphocytes from the duodenum were quantified by immunohistochemistry assays. Samples were processed as indicated above, except that sections were washed in 0.05% PBS-T and later incubated for 1 h in a humidified chamber at RT with one of the following: biotynilated rat anti-mouse monoclonal antibodies diluted in 1% bovine serum albumin–PBS: anti-mouse CD4 (BD Pharmingen, USA, 553728), anti-mouse CD8 (BD Pharmingen, USA, 553029), anti-mouse/human CD45R (B220) (eBiosciences, USA, 13-0452), or monoclonal rat anti-mouse F4/80 (Caltag Lab, USA, RM2915). After washing, sections were incubated for 1 h in a humidified chamber at RT with horseradish peroxidase (HRP) streptavidin conjugated (Zymed, USA; 43-8323). After washing again, the activity of peroxidase was revealed with H_2O_2 diaminobenzidine (Pierce, USA). The procedure for control groups included omission of the primary antibody as well as its replacement with normal rat serum. Samples were counterstained with Harris' hematoxylin, then dehydrated and covered with synthetic resin (Merck, Germany).

Cells were quantified in random fields at a magnification factor of 400 by using the image analysis program Imagen-Pro Plus 5.1. Fields were adjusted to give the number of cells per 0.05 mm^2 of tissue. Tissue sections were analyzed by two independent observers.

2.5. ELISA for IgA

We determined IgA antibody concentration in fluids from the proximal small intestine using ELISA. Ninety-six microwell polystyrene plates (Corning) were coated with 100 μl of rabbit anti mouse IgA (20 μg/ml) and incubated for 18 h at 4°C. Thereafter they were washed three times with PBS-T, then blocked by adding 100 μl of PBS-T with 10% non-fat milk. After incubating for 1 h at 37°C, the plates were washed four times with PBS-T. One hundred microliters of intestinal fluid (diluted 1:2) were added. The plates were incubated for 18 h at 4°C, then washed 5 times with PBS-T and 5 times with PBS. One hundred microliters of freshly diluted (1:3000) HRP-labeled goat anti mouse IgA were added to each well. The plates were incubated during 2 h at 37°C and later washed 5 times with PBS-T. Finally, 100 μl of the substrate solution (0.1% H_2O_2 plus 0.1% orthophenylenediamine in citrate phosphate buffer) were added to each well. After 15 min the reaction was stopped with 2.5 M of sulfuric acid and the optical density in each well was determined at 492 nm using a microplate reader. The concentration of *serum IgG* was measured using a sandwich ELISA, as previously described, except that the microplates were coated with 100 μl of rabbit anti-mouse IgG (50 μg/ml).

2.6. Detection of alpha chain and pIgR proteins by chemiluminescent Western blotting

A 4-cm segment from the duodenum was homogenized with 1 ml of RIPA lysis buffer plus a protease inhibitor cocktail (Roche Diagnostics, USA, 11836145001), and stored at −80°C for protein analysis. The protein content was quantified by the Lowry method. Soluble protein was denatured by immersing it in boiling water for 10 min with sodium dodecyl sulfate and β-mercaptoethanol; 50 μg of total protein were separated in a denaturing 10% polyacrylamide gel by electrophoresis at 88 V for 2 h. The proteins were transferred to a polyvinylidene fluoride membrane at 18 V for IgA and 20 V for pIgR during 1h in the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The membranes were blocked with 5% (w/v) nonfat dry milk in Tris buffer (pH 7.4) containing 0.1% (v/v) Tween 20 for 2 h at room temperature with constant agitation. They were then incubated overnight at 4°C with constant agitation by using one of the following antibodies: (a) *rabbit polyclonal serum* produced against α-chain purified from human IgA at a dilution 1:10,000; or (b) rabbit polyclonal serum produced against a peptide of secretory component (H-GNSVSITYYPPTSVNRHTRKYWCOH) at a dilution 1:200. Membranes were washed and incubated with goat anti-rabbit-IgG HRP (Invitrogen, Carlsbad, CA, USA, G21234) at a 1:10,000 dilution for 2 h at room temperature with constant agitation. After the last wash, the membranes were incubated for 1 min with Western Blotting Luminol Reagent (Santa Cruz) and bands were detected using photographic film (Amersham Hyperfilm ECL) with an exposure of 30 s for IgA and 2 min for pIgR. Densitometric measurements of protein bands were analyzed and quantified with the Quantity One 1-D Analysis Software.

2.7. Real-time polymerase chain reaction (RT-PCR) assays

2.7.1. RNA extraction

Total RNA from the duodenal mucosa was isolated using TRIzol reagent according to the manufacturer's protocol (Life Technologies, USA). Isolated RNA was quantified using the GENESYS 10 Series spectrophotometer (Thermo Scientific, USA) and 5 μg of RNA were separated on a 1.0% agarose gel containing ethidium bromide in MOPS buffer. Running buffer and gel contained 0.2 M formaldehyde. To avoid trace amounts of DNA contamination, RNA samples were treated with amplification grade DNase I (Invitrogen) before reverse transcription. All RNA samples were stored at −70°C in RNA elution solution until further use.

2.7.2. DNA synthesis and RT-PCR assays

We used 0.5 μg of RNA for reverse-transcription with random hexamers in 20-μl reaction volume using Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). The reactions were performed in Eppendorf Mastercycle thermocycler (Eppendorf, México, Distrito Federal, México). The amplified cDNA was quantified on a photometer at 260nm. RT-PCR reactions were carried out using the Mouse Universal ProbeLibrary (Roche Diagnostics). Specific oligonucleotide primers were originally generated by using the online assay design software (ProbeFinder: http://www.universal-probelibrary.com) and the primer sequence for each gene that is shown in Table 1. The 20-μl reaction mixture contained 1×LightCycler TaqMan Master reaction mixture (Roche Diagnostics), 200 nM of each primer, 100 nM of Universal ProbeLibrary probe, 0.5 U LightCycler Uracil-DNA Glycosylase and 2 μl of standard DNA in appropriate dilution. The amplification was performed in borosilicate glass capillaries (Roche Diagnostics). The RT-PCR assay included a standard curve of four serial dilution points for each gene and samples were normalized with the endogenous control 18S.

Table 1
Primer sets used for RT-PCR; designs are based on ensemble transcript ID of the mouse probe library

Gene/ensemble transcript ID	Forward primer 5′-3′	Reverse primer 5′-3′
IgA/04692101001	cgtccaagaattggatgtga	agtgacaggctgggatgg
IFN-γ/04686942001	tctggaggaactggcaaaag	ttcaagacttcaaagagtctgagg
IL-10/04686942001	actgcacccacttcccagt	tgtccagctggtccttttgtt
IL-12β/04692110001	atccagcgcaagaaagaaaa	ctacgaggaacgcacctttc
IL-2/04685148001	gctgttgatggacctacagga	ttcaattctgtggcctgctt
IL-4/04692250001	catcggcattttgaacgag	acgtttggcacatccatctc
IL-6/04685032001	gctaccaaactggatataatcagga	ccaggtagctatggtactccagaa
pIgR/04688635001	agtaaccgaggcctgtcctt	gtcactcggcaactcagga
TGF-β1/04688953001	tggagcaacatgtggaactc	gtcagcagccggttacca
TNF/04686993001	ctgtagcccacgtcgtagc	ttgagatccatgccgttg

Data were analyzed by LightCycler Software Version 4.0 by using the cycle threshold (Ct) to known relative levels of mRNA. The amount of each gene and 18S mRNA in samples was estimated with standard curves representing the log of the input amount (log starting cDNA molecules) as the *X*-axis and the threshold cycle as the *Y*-axis. A relative standard curve for RT-PCR was used as a standard set of samples that linked the experimental polymerase chain reaction (PCR) capillaries together and permitted overall analysis of the samples. Preparation and utilization of this standard curve as a quality control of the efficiency of amplification of PCR has been described [20].

2.8. Corticosterone and norepinephrine assays

Serum corticosterone and norepinephrine levels from individual mice were determined using commercially available costicosterone (Assay designs, Ann Arbor, MI, USA, Cat. no 901-097), and epinephrine (Alpco Immunoassays, Salem, NH, USA, cat. No. 17-Epihu-E01) EIA kits. The corticosterone and norepinephrine concentration in the serum samples was calculated from a standard curve and expressed in nanograms per milliliter.

2.9. Statistical analysis

Data are presented as the mean±S.D. The comparison of two groups was analyzed by using the Student's unpaired two-tailed *t* test. All analyses were performed using the statistical program Sigma Stat for Windows Version 2.03 software (SPSS).

3. Results

3.1. CR reduced intestinal IgA levels and the number of IgA$^+$ cells in the LP

The intestinal IgA concentration was slightly but significantly lower in CR than AL mice (Fig. 1). The effect of CR on systemic humoral immune response was analyzed by measuring the levels of IgG, the main serum immunoglobulin. CR reduced the serum levels of IgG (Fig. 1), indicating that CR affects both the mucosal and systemic humoral immune response.

To assess whether or not the decrease in intestinal IgA levels resulted from a lower quantity of IgA secreting cells in the LP, the amount of IgA$^+$ cells was determined. A significantly lower number of IgA$^+$ cells was found in CR than AL mice (Fig. 2; $P<.05$). CR did not have a significant effect on the number of B$^+$ cells, CD4$^+$ T, CD8$^+$ T cells, and macrophages in the LP (Fig. 2), or on the histological structure of the villus (Fig. 3). These results show that the decrease in intestinal IgA levels in CR mice is probably a consequence of the lower number of IgA$^+$ cells in the LP.

3.2. Caloric restriction increased the expression of α-chain, and pIgR mRNA in the small intestine and reduced such expression in the liver

Because the expression of the genes for α-chain (IgA), and the pIgR in the intestinal mucosa is important for IgA secretion to the intestinal lumen [21,22], we analyzed the expression of these genes in the intestinal mucosa by RT-PCR. Caloric restriction caused a significant increase in the expression of the genes for α-chain, and the pIgR ($P<.001$, and $P<.05$, Fig. 4A).

Given that in mouse most of the S-IgA in the proximal intestine is derived from the hepatobiliary transfer of pIgA [22,23], we determined the expression of alpha–chain, and pIgR genes in the liver of CR and AL mice (Fig. 4B). Caloric restriction caused a significant decrease in the expression of the genes for α-chain and pIgR ($P<.001$), and probably in content of S-IgA in the bile.

3.3. Caloric restriction modified the synthesis of IgA and pIgR in the proximal intestine

Because RT-PCR data alone are not sufficient to draw conclusions, we quantified the synthesis of the alpha chain and the pIgR by Western blotting. There was a significantly lower synthesis of alpha chain ($P<.001$) but a higher synthesis of pIgR ($P<.05$) in duodenal mucosa of CR mice (Fig. 5). The higher expression of the gene for pIgR correlated with a higher synthesis of the corresponding protein. However, the higher expression of the gene for alpha chain was not associated with a higher synthesis of the IgA in the whole duodenal mucosa.

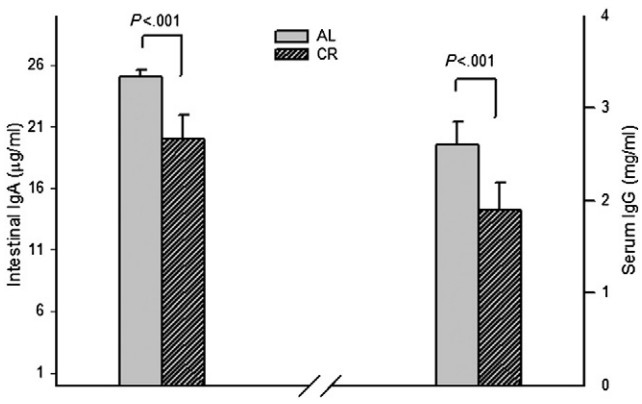

Fig. 1. Effect of CR on the intestinal IgA. After a 6-h fasting period, the mice were sacrificed and the intestinal fluid was obtained. The intestinal IgA and serum IgG concentration was determined by ELISA, and is expressed as micrograms per milliliter and milligrams per milliliter, respectively. Data were obtained from eight mice per group and are presented as the mean±S.D. CR reduces the concentration of intestinal IgA and serum IgG compared with the AL group ($P<.001$, Bonferroni *t* test). Similar results were obtained in two independent experiments.

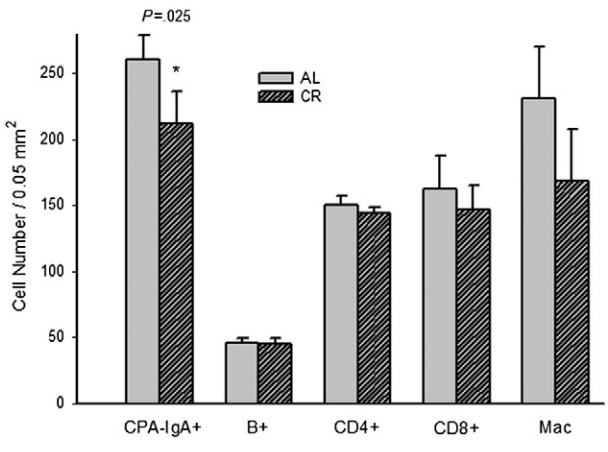

Fig. 2. Effect of CR on the number of IgA$^+$ cells, B and T lymphocytes, and macrophages. CR and AL mice were killed, and the duodenal tissue samples were obtained. The number of plasma cells containing IgA (IgA+cells), B and T lymphocytes, and macrophages was quantified by immunohistochemistry with a specific antibody. The number of cells is expressed as cells per 0.05 mm^2. Data were obtained from four to eight mice per group and are presented as the mean±S.D. The statistical analysis was performed using a nonpaired Student's *t* test. CR reduced the basal number of IgA$^+$ cells ($P=.025$), but did not change the number of B, T CD4$^+$ and T CD8$^+$ cells.

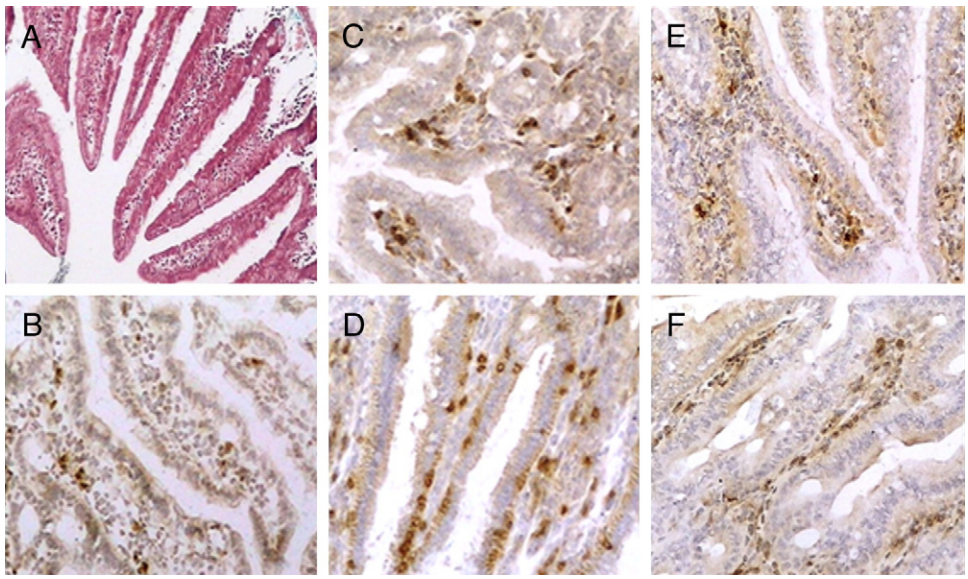

Fig. 3. Histology and immunostaining of immune cells in the duodenum of CR mice. (A) The basic structure of the villi is normal in the duodenum of CR mice (Gomori; original magnification ×100). (B) Macrophages F4/80+. (C) CD4+ T lymphocytes. (D) CD8+ T lymphocytes. (E) IgM+ B lymphocytes. (F) IgA+ plasma cells (original magnification ×200).

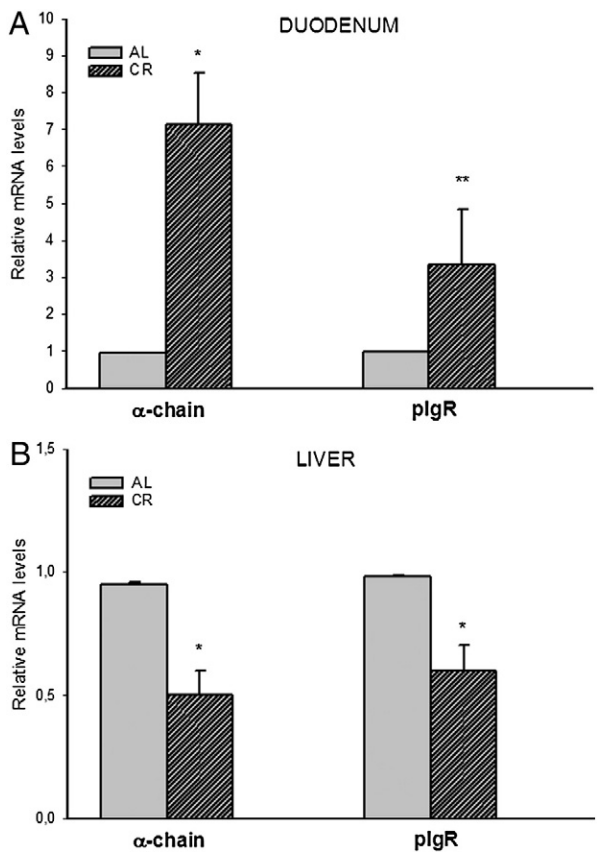

Fig. 4. Real-time RT-PCR analysis of α-chain and polymeric Ig receptor (pIgR) in duodenum (A) and liver (B) samples of CR mice. Samples of the duodenal mucosa and liver from CR mice were collected and the mRNA expression of the α-chain and pIgR was measured by real-time RT-PCR, as detailed in materials and methods. mRNA levels were calculated by using the comparative parameter threshold cycle (C_t) method and normalized to ribosomal RNA. Data represent the means±S.D. (n=6–10). CR significantly increased pIgR and alpha chain mRNA levels in the duodenum (*P<.001 and **P<.05), but decreased such levels in the liver (*P<.001).

3.4. Caloric restriction alters the expression of genes for several cytokines

Since the expression of alpha-chain and pIgR mRNA is up-regulated by cytokines that mediate innate and adaptive immunity [14,24–27], we quantified the expression of genes for TNF-α, IL-6, IL-10, IL-12, IL2, IL-4, IFN-γ and tumor growth factor (TGF)-β, in the proximal intestinal mucosa and the liver by using RT-PCR. Whereas CR significantly increased the expression of the genes encoding for TNF-α and IL-10 (P<.001 and P<.05; Fig. 6A), as well as for IFN-γ and TGF-β (P<.001, Fig. 6B), it decreased the expression of the gene for IL-2 (P<.05; Fig. 6B). No change was found in the expression of the genes for IL-4, IL-6 and IL-12 in the duodenal mucosa.

With CR, the patterns of mRNA expression of cytokines, both of the innate and adaptive immunity, were different in the proximal small intestine and liver. Whereas CR significantly increased the expression of the genes encoding for IL-10 and TGF-β in the intestine (Fig. 6), it decreased such expression in the liver (Fig. 7). On the other hand, CR

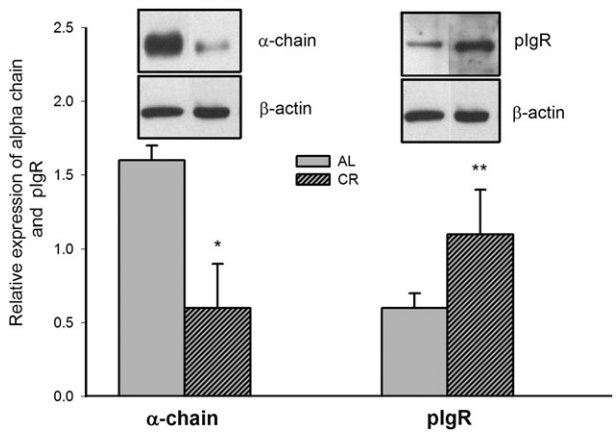

Fig. 5. Western blot analysis of alpha-chain and . Western blot analysis of alpha-chain and pIgR in duodenal mucosa. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis was performed as described in Section 2. Western blot detected bands at ~68 KDa (alpha-chain), and ~80 kDa (pIgR) (insert). The alpha-chain expression was lower whereas the pIgR expression was higher in the duodenum of CR mice. Densitometric values are the mean±SD (n=5) and are normalized for β-actin.

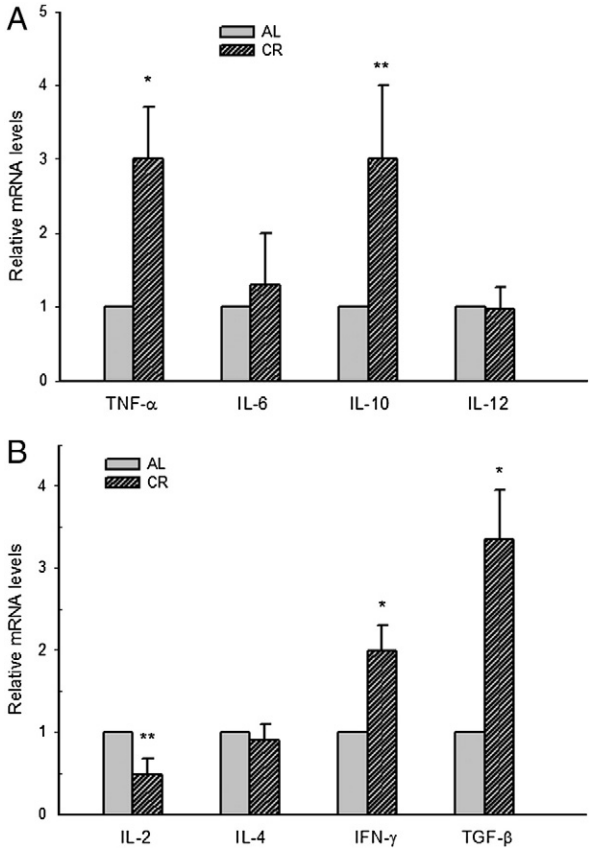

Fig. 6. Real-time RT-PCR analysis of cytokines in duodenal samples of CR mice. (A) mRNA levels of cytokines of the innate immunity. (B) mRNA levels of cytokines of the adaptive immunity. Samples of the duodenum of CR mice (n=6–10) were processed as detailed in Materials and Methods. The mRNA levels for TNF-α, IL-10, IFN-γ and TGF-β were higher in CR mice. Contrarily, IL-2 mRNA levels were lower in CR than AL mice (*P<.001 and **P<.05).

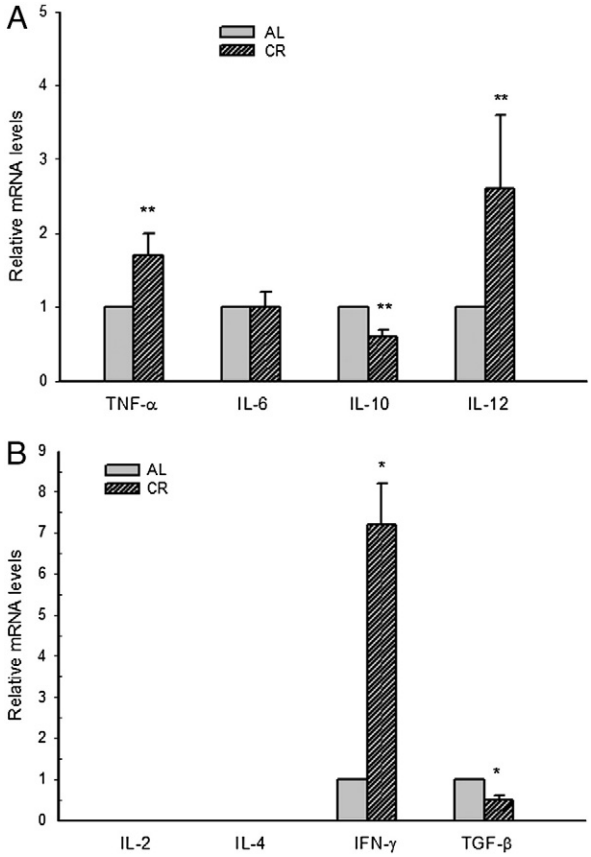

Fig. 7. Real-time RT-PCR analysis of cytokines in liver samples of CR mice. (A) mRNA levels of cytokines of the innate immunity. (B) mRNA levels of cytokines of the adaptive immunity. Samples of the whole liver from mice with CR (n=6–10) were processed as detailed in Materials and Methods. The mRNA levels for TNF-α, IL-12, and IFN-γ were higher in CR mice. Contrarily, IL-10 and TGF-β mRNA levels were lower in CR than AL mice (*P<.001 and **P<.05).

increased the expression of the genes encoding IL-12 and IFN-γ in the liver (Fig. 7) but not in intestine. Finally, CR increased the expression of the gene for TNF-α in both the intestine and liver.

3.5. Caloric restriction did not alter serum levels of corticosterone and noradrenaline

Since glucocorticoids and catecholamines regulate the synthesis of IgA and pIgR [28-30], we determined serum corticosterone and norepinephrine concentrations in CR and AL mice (Table 2). Since CR did not modify corticosterone and norepinephrine concentrations, these hormones do not mediate the observed effects of this dietary regimen.

4. Discussion

The most relevant aspect of the current contribution is that it shows, for the first time, that CR decreased intestinal IgA levels in mice. The observed decrease in serum IgG with CR evidences the effect of this regimen on the systemic humoral as well as intestinal immune responses. On the contrary, in another study with CR an increase was observed in the number of IgM and IgG cells in spleen and serum immunoglobulins (IgG and IgM) in mice and monkeys [31-33] These apparently contrary results could be due to the different type of dietary protocols employed. Whereas Ebersole et al. [31] used an energy-restricted high fat diet or a continuous long-term calorie-restricted diet, we used an alternate day calorie restriction diet.

The decrease in intestinal IgA levels must be explained in relation to the two principal mechanisms for intestinal IgA production and secretion: (i) the production and secretion of pIgA by IgA^+ cells in the LP, and (ii) the transport of the pIgA-pIgR complex across epithelial cells, where it is then cleaved and secreted into the lumen as S-IgA [22]. Therefore, we analyzed the number of IgA-containing cells in the LP as well as the expression of the genes for alpha-chain of IgA, and the pIgR. We also analyzed the synthesis of the alpha-chain and pIgR proteins by Western blotting.

The reduced number of IgA-producing cells in the LP of CR mice indicates that the first mechanism was at least in part responsible for the decreased intestinal IgA levels. On the other hand, CR increased the expression of the genes for alpha-chain of IgA, and pIgR, which could increase both the synthesis and transport of pIgA.

The increased expression of the gene for alpha-chain suggests that individual plasma cells produce more polymeric IgA, since the production of pIgA depends on the synthesis of alpha chains [22].

Table 2
Effect of CR on serum corticosterone and norepinephrine levels

	CORT (ng/ml)	NE (ng/ml)
CR	17.2±10	6.4±3
AL	24.4±16	7.4±2.

Peripheral blood was collected from CR and AL mice and subjected to corticosterone (CORT) and norepinephrine (NE) assays. Data are mean±S.D. values from seven to 10 mice. Corticosterone and norepinephrine levels were similar in both groups (P>.05, Student's t test).

However, the higher production of pIgA by each plasma cell did not counteract the reduction of pIgA caused by the lower number of plasma cells in the lamina propria.

The mechanism by which CR modifies the transcription of alpha-chain is not known. The increase in the alpha-chain expression probably is related to the higher expression of TGF-β (see below), which is considered an essential IgA switch factor, and to transcription factors such as CBFa3 [22,34]. Although the higher expression of the gene for the alpha chain was not associated with a higher synthesis of IgA in the duodenal mucosa as a whole, it is possible that the lower number of plasma cells in CR mice produced more IgA per cell than in AL mice. Also, the reduced concentration of IgA in duodenum could be due to instability and a rapid degradation of alpha-chain mRNA. On the other hand, there was both a higher expression of the gene for pIgR and a higher synthesis of the corresponding protein in CR mice.

Overall, the results clearly show that the second mechanism (transport of the pIgA-pIgR complex across epithelial cells) was not related to the decrease in the intestinal IgA. Apparently, the reduced production and secretion of IgA was caused by the reduction in the number of IgA^+ cells in the LP, and a lesser synthesis of polymeric IgA.

A probable additional mechanism by which CR reduced IgA levels in the proximal intestine is by reducing the content of S-IgA in the bile, which discharges into the proximal intestine. We found that CR decreased the expression of genes for the α-chain and pIgR ($P<.001$) in the liver, which may have two consequences: a reduction in the hepatobiliary transfer of circulating pIgA mediated by pIgR in hepatocytes, and a reduction in the synthesis of pIgA by plasma cells that populate the biliary tree [22,23].

In summary, the decrease in the intestinal IgA levels is probably related to a reduced synthesis of IgA in the lamina propria and a reduced secretion of S-IgA into the bile. Independently of the mechanisms that cause a reduction of intestinal IgA levels, this effect may increase the susceptibility of CR mice to infections by intestinal pathogens as well as to colonization by commensal microbiota [15,35].

Although it is considered that severe protein-energy malnutrition does not affect terminal differentiation of B cells [36], this condition does lower the number of intestinal IgA- and IgM-containing cells [36]. The mechanism by which CR reduces the number of IgA plasma cells in the LP of the small intestine is still unclear.

Since CR did not affect the populations of B lymphocytes or $CD4^+$ and $CD8^+$ T lymphocytes, the reduction of IgA^+ cells was caused by a lower migration of B cell precursors to the IgA^+ cells in the LP, or a lower number of T cells involved in IgA class switching and terminal differentiation. One possible mechanism is that CR inhibits the terminal differentiation of plasma cells in the LP, in which case cytokines would play an important role [24,27]. Accordingly, it has been reported that in vitro CR suppresses IgA production, which is induced by both Th-1 and Th-2 cytokines in cells of the salivary gland of young mice [37].

Since to the best of our knowledge there have been no reports about the expression of genes encoding for cytokines in the intestinal mucosa of mice under CR, we consider it important to discuss the influence of this diet on the resulting mix of cytokines, and the consequent effect on IgA production. CR did not modify the expression of the genes for IL-4, IL-6, or IL-12, but it reduced the expression of the gene for IL-2, and increased the expression of the genes for TNF-α, IL-10, IFN-γ and TGF-β. The higher expression of genes for TGF-β and IL-10, which regulate the production of IgA, could be responsible for the observed increase in the expression of the gene for α-chain by plasma cells in the intestinal mucosa.

The CR induced increase in the expression of the gene for pIgR and the synthesis of the respective protein in the intestinal mucosa is in contradiction to the results reported in another study, where CR was found to reduce the expression of the pIgR in submandibular glands of mice [10]. This discrepancy is probably related to the tissue specific regulation of pIgR expression [28,38]. Because CR upregulates the expression of the genes for TNF-α, IFN-γ, and TGF-β, these cytokines are probably responsible for the increase in the expression of the pIgR in CR mice, given that these same cytokines enhance the expression of the pIgR in cell culture systems [14,25,39].

The higher expression of the gene for pIgR under conditions of CR could be due in part to the effect of glucocorticoids and catecholamines [40-44]. However, we did not find any change in the levels of corticosterone and norepinephrine in CR mice, and therefore the changes that occurred in the synthesis of α-chain and pIgR in the present study cannot associated with those hormones.

CR also affected mRNA levels of several cytokines in the liver, a lymphoid organ that contains a diverse population of lymphoid and accessory cells, as well as important functional relationships with the intestine [45]. However, the patterns of mRNA expression were different in the intestine and liver, probably due to some of the distinguishing characteristics of the hepatic lymphoid cells [45,46]. It is likely that CR also differentially modified the expression of cytokines in other lymphoid tissues such as the spleen or lymph node, a question that is currently under study. It can be seen that CR affects mucosal and systemic immune responses, although in a different way.

In conclusion, CR decreased the levels of IgA in the intestine, apparently as a consequence of two mechanisms: (a) a reduced number of IgA^+ cells in the lamina propria and therefore a decrease in the production and secretion of this immunoglobin, and (b) and reduced secretion of S-IgA into the bile that discharge into the proximal intestine. Contrarily, CR increased the expression of the gene for pIgR and its synthesis, indicating that transport of IgA was not a factor in the decrease of S-IgA. Additionally, CR modified the expression of the genes for IL-2, IL-10, TNF-α, IFN-γ and TGF-β, all of which regulate the synthesis of IgA and pIgR, the inflammatory response, and the immune response in the intestine. Further research is needed to determine the influence of the complex balance of cytokines in the changes produced by CR on IgA levels and the overall effect of the immune response.

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