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Yogurt feeding inhibits promotion and progression of experimental colorectal cancer

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Alejandra de Moreno de LeBlanc[1,2], **Gabriela Perdigón**[1,2]

[1] Cátedra de Inmunología. Instituto de Microbiología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina
[2] Centro de Referencias para Lactobacilos (CERELA), Chacabuco, Tucumán, Argentina

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Summary

Background: In BALB/c mice, a yogurt diet given before and after the carcinogen 1, 2 dymethylhydrazine (DMH) inhibited colon cancer. This paper studied at which stage of tumor development (initiation, promotion or progression) yogurt exerts its antitumor activity.

Material/Methods: Six experimental groups were used: 1) non-treatment control; 2) DMH control; 3) yogurt-DMH-yogurt: yogurt administered before and after DMH. 4) yogurt-DMH: yogurt given only 10 days before DMH; 5) DMH-yogurt: yogurt given cyclically after DMH; and 6) yogurt control. The groups DMH-yogurt and yogurt-DMH were compared histologically and TNFα, INFγ, IL-10 and IL-4 cytokines, $CD4^+/CD25^+$ T cells, and apoptotic cells were determined in large intestine biopsies. TNFα and INFγ were also determined in cells isolated from large intestine nodules and from Peyer's patches.

Results: The DMH-yogurt group did not develop tumor. The yogurt-DMH group showed only tumor delay; TNFα, INFγ and IL-10 increasing in this group in all the periods assayed. These results agree with those already reported for DMH control and yogurt-DMH-yogurt. There was no correlation between the high levels of IL-10 and $CD4^+/CD25^+$ T regulatory population. IL-4 and apoptotic cells increased in the yogurt-DMH group only in the first months. In the DMH-yogurt group, cellular apoptosis increased during the whole treatment. Yogurt feeding induced TNFα and INFγ increases in cells isolated from large intestine nodules. These cytokines also increased in cells from Peyer's patches of the yogurt control group.

Conclusions: These results show that yogurt inhibited tumor progression and promotion by modulating the immune response and stimulating cellular apoptosis.

key words: yogurt • colon cancer • immune response

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Author's address: Gabriela Perdigón, Centro de Referencias para Lactobacilos (CERELA). Chacabuco 145, (4000) Tucumán, Argentina, e-mail: perdigon@cerela.org.ar

BACKGROUND

The normal microbial colonization of the mammalian intestine contributes to the normal development of the gut mucosal immune system. Probiotics can be added to the diet to improve the intestinal microbial balance and the intestine immunologic barrier, particularly through immunoglobulin A response and alleviation of intestinal inflammatory responses, which produce a gut-stabilizing effect [1–2]. Lactic acid bacteria (LAB) are present in many foods such as yogurt and are frequently used as probiotics to favor some biological functions in the host. Yogurt is fermented milk that contains the *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* as its starter. Many investigators have studied the therapeutic effects of yogurt and LAB commonly used in yogurt production against diseases such as cancer, infection, and gastrointestinal disorders. The immunomodulating and immunostimulating properties of yogurt and fermented milks have also been well documented [3–5]. Very much attention has been focused on decreasing cancer risk through dietary alterations, particularly by increasing the intake of dietary fiber (including prebiotics) and consumption of probiotics. *In vivo* and *in vitro* studies have shown that the growth of transplantable and chemically induced tumors was inhibited by yogurt and other LAB (6–8). *L. acidophilus* reduced the risk of carcinogenesis in a chemically induced colon cancer [9], and *L. casei* inhibited a chemically induced murine fibrosarcoma [10]. A series of studies examining the influence of bifidobacteria and/or fructooligosacharides (prebiotic) on the presence of aberrant crypts and/or tumor in the colon were reported by Reddy and collages [11,12].

Yogurt may reduce the risk of carcinogenesis either by reduction of the carcinogen itself or by reduction of the enzymes that promote the conversion of precarcinogen to carcinogen [13]. Another mechanism involves tumor suppression by enhancement of immune response [5]. In an experimental model using BALB/c mice it was demonstrated that cyclical yogurt feeding inhibited a chemically induced colon tumor. In these animals, a large inflammatory immune response was observed during tumor development. Yogurt feeding inhibited tumor development by decreasing the inflammatory immune response and increasing the number of IgA-secreting cells and $CD4^+$ T lymphocytes in the lamina propria of the large intestine [8]. Production of cytokines such as TNFα, which favors apoptosis mechanisms, or IL-10, was also observed [14]. Yogurt feeding itself was related with increased levels of some cytokines, such as TNFα, IFNγ and IL-10. Berg et al. [15] studied the role of IL-10 in intestinal inflammation and carcinogenesis. Mice with a disruption in the IL-10 gene showed inflammatory changes in the cecum, colon and rectum, with high incidences of colorectal adenocarcinomas. IL-4 was reported together with IL-10 as a potent inhibitor of Th-1 effector function *in vivo* controlling deleterious Th1-mediated inflammatory responses that occur in some infectious and autoimmune diseases [16], and it was also related with immune modulation in some tumor models [17].

We analyzed the anti-inflammatory activity of yogurt compared with indomethacin [18]. It was suggested that this activity could be responsible for its antitumor effect. It is known that the development of colon cancer presents a sequence of events that occurs in definable steps (initiation, promotion, and progression). The aim of the present work was to study in which of these levels of tumor development yogurt exerts its antitumor activity to gain a better understanding of the beneficial effects of yogurt on colon cancer.

MATERIAL AND METHODS

Animals and diets

BALB/c mice, weighing 25–30 g, were obtained from the random-bred closed colony kept in our Microbiology Department. The mice were separated into six experimental groups: 1) DMH group: the mice received only injections of 1–2 dimethylhydrazine (DMH) to induce tumor growth; 2) yogurt-DMH-yogurt group: the mice were fed with yogurt for ten consecutive days (basal yogurt), treated with DMH, and then fed again cyclically with yogurt; 3) yogurt-DMH group: the mice were given yogurt for 10 consecutive days and then treated with DMH to study the effect of yogurt on tumor initiation; 4) DMH-yogurt group: the mice were treated with DMH and fed cyclically with yogurt to analyze the effect of yogurt on tumor progression, receiving no basal yogurt feeding before the drug; 5) yogurt control group: mice were fed cyclically with yogurt from the eighth week of the experiment until the sixth month to analyze the long-term effect of yogurt administration; and 6) the non-treatment group: mice not given any specific treatment. All groups were fed *ad libitum* with a balanced diet. Each experimental group consisted of 30–35 mice.

Yogurt preparation

Simulated commercial yogurt was freshly prepared and controlled every day to keep the number of bacteria constant and to avoid variations due to storage. The yogurt was prepared from cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (strain pools were used from the CERELA culture collection). The total number of bacteria in the fermented product was 2×10^9 cells/ml.

Tumor induction and feeding procedure

To induce colon tumor, mice were injected with the carcinogen DMH dihydrochloride (Sigma, St. Louis, USA). Each mouse received, subcutaneously, 20 mg DMH/kg body weight in 0.1 ml of saline solution containing 1.5 g/l of EDTA, pH 6.4, weekly during 10 weeks. These animals developed tumor five or six months after the first injection (tumor control or DMH group).

Other test mice were given a diet supplement with 3 ml of yogurt for 10 consecutive days (basal yogurt). At the end of the feeding period they were separated into different groups: 1) Each mouse was injected with DMH as in

the tumor control group, but in the eighth week, yogurt was added to the diet for ten consecutive days, followed by a one-week break, and then again yogurt feeding for ten days. Feeding was given in this manner cyclically until the sixth month (yogurt-DMH-yogurt group). 2) The mice were injected with DMH as described above and were not fed with yogurt again (yogurt-DMH group). 3) The mice were not injected with DMH, and yogurt feeding was repeated cyclically from the eighth week until the sixth month (yogurt control group).

The last test group was injected weekly with DMH for ten weeks without basal yogurt feeding. In the eighth week, yogurt was given for ten days and then cyclically as with the other groups until the sixth month (DMH-yogurt group).

Histological studies

Mice were killed monthly by cervical dislocation. The large intestine was removed and washed with a physiological solution (0.85% NaCl). Tissues were prepared for histological evaluation using the method described by Sainte Marie [19]. Serial paraffin sections of 4 μm were made. Sections from the yogurt-DMH and DMH-yogurt groups were stained with hematoxilin-eosin for light microscopy examination.

Cytokine-producing cell determination in histological sections

Tissue sections from the different groups were used for immunofluorescence assays. $IL4^+$ cells were studied in all the groups of mice, IFNγ- and TNFα-positive cells were studied in the yogurt-DMH group, and IL-10-positive cells in the yogurt-DMH and yogurt-DMH-yogurt groups. Cytokines were detected by indirect immunofluorescence. After deparaffinization and rehydration in a graded series of ethanol, paraffin sections (4 μm) were incubated with a 1% blocking solution of BSA-HBSS (bovine serum albumin-Hank's balanced saline solution) for 30 min. They were then washed in saponin-HBSS and incubated with normal goat serum (diluted 1: 50) for 30 min. Rabbit anti-mouse TNFα, IFNγ, IL-10, and IL-4 (Peprotech, Inc. Rocky Hill, NJ, USA) polyclonal antibodies (diluted in saponin-HBSS) were applied to the sections for 75 min at RT (room temperature). This incubation was followed by two washes with saponin-HBSS. The sections were then treated with a dilution of the goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC, Jackson Immuno Research, Labs. Inc, West Grove USA) for 45 min at RT, and washed in saponin-HBSS.

The number of fluorescent cells was counted in thirty fields of vision as seen at 1000× magnification using a fluorescence light microscope. The results were expressed as the number of positive cells in ten fields of vision.

Isolation of mononuclear cells from Peyer's patches

The small intestine of each mouse from the yogurt control and non-treatment groups was removed and the Peyer's patches were excised in HBSS with FBS (fetal bovine serum) added. The epithelial cells were separated with an HBSS/FBS solution containing EDTA, stirring with a magnetic agitator. The mononuclear cells (sediment) were incubated in an HBSS/FBS/PROTEASE/DNase solution (enzymes from Sigma, St. Louis, USA). The lymphocytes collected from supernatant were washed with HBSS and then with RPMI 1640 medium (Sigma, St. Louis, USA). These cells (total cells) were adjusted to 4×10^6–5×10^6 cells/ml in RPMI 1640. The adherent cells were separated from the whole population using their adherence property on glass slides. Non-adherent cells were harvested and their concentrations adjusted to 4×10^6–5×10^6 cells/ml in RPMI 1640. 20 μl of total and non-adherents cells were placed in each well of a immunofluorescence slide. All cellular populations were fixed with formalin (ICC fixation buffer, PharMingen).

Isolation of mononuclear cells from infiltrative nodular tissue of the large intestine

To isolate mononuclear cells from the lymphoid nodule, the large intestine of each mouse from the DMH and yogurt-DMH-yogurt groups was removed, washed and examined. The nodules were excised in HBSS with added FBS, gentamycin and fungizone. Mucus was removed with 1 mM dithiotreitol, stirring with a magnetic agitator. After washes with HBSS, the epithelial cells were separated with an HBSS/FBS solution containing EDTA with a magnetic agitator. The sediment (mononuclear cells) was incubated in a solution composed of RPMI-1640, FBS, collagenase, DNase and hialuronidase to produce a single cell suspension (enzymes were obtained from Sigma, St Louis, USA). The cells were washed with RPMI-1640. The different cellular populations (total, adherent, and non-adherent cells) were separated as described previously and fixed with formalin.

Cytokine determination in the isolated cells

TNFα and IFNγ were determined in the cells fixed with formalin. These were incubated with 1% blocking solution of BSA/PBS, washed with PBS, and incubated with normal goat serum (diluted 1/10). The activity of the endogenous peroxidase was blocked with an H_2O_2/methanol solution. The cells were then incubated with an avidin- and biotin-blocking solution (Avidin/biotin blocking kit, Vector laboratories, Inc, Burlingame, USA) to block the endogenous avidin and biotin. The cells were incubated with rat anti-mouse TNFα or IFNγ polyclonal antibody (diluted in diluents ICC cytokine buffer, PharMingen), washed with PBS, and incubated with a biotin-conjugated goat anti-rat Ig-specific polyclonal antibody (Pharmingen International, Becton Dickinson Company). Vectastain *Elite* ABC solution (Vector Labs, Burlingame, USA) was added to the cells and incubated with 3, 3 diaminobenzidine tetrahydrocloride (Sigma, St Louis, USA). The results were expressed for the adherent cells as the percentage of positive cells (counted at 1000× magnification in light microscopy) and for non-adherent and total cells as the number of positive cells in a well (8×10^4 cells) counted at 1000× magnification.

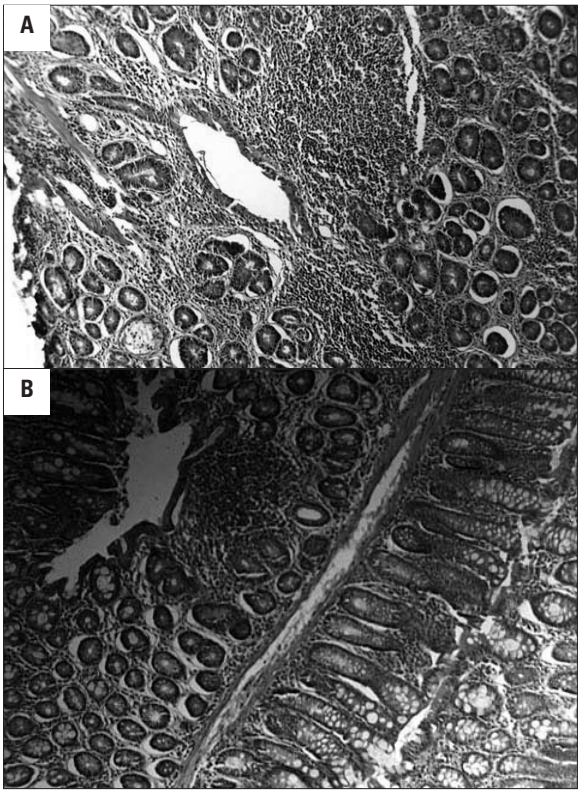

Figure 1. Light micrograph of hematoxilin-eosin stained sections from the large intestine of mice fed with yogurt before or after tumor induction. (**A**) The yogurt-DMH group in the fourth month after starting DMH injection (100×). Inflammatory infiltrates extend between glands, crypt abscesses, lack of tissue architecture normal. (**B**) The DMH-yogurt group at the end of treatment (7th month). Note the cellular increase in the lamina propria and abundance of mucin in goblet cells. Tissue organization conserved as in the no-treatment control. (magnification 100×).

Immunofluorescence assay for CD25$^+$ and CD4$^+$ cells

CD25$^+$ cells were analyzed comparatively with CD4$^+$ cells in samples of large intestine of mice in the yogurt-DMH and yogurt-DMH-yogurt groups that showed increase number of IL-10 positive cells. The number of CD25$^+$ or CD4$^+$ cells was measured with a direct immunofluorescence assay. Monoclonal antibodies conjugated with FITC were used (Sigma, St. Louis, USA).The number of fluorescent cells was counted in thirty fields of vision as seen at 1000× magnification using a fluorescence light microscope. The results were expressed as the number of positive cells in 10 fields of vision.

Apoptosis determination

Apoptosis was evaluated for the presence of DNA breaks detected in paraffin sections using the Apoptosis Detection System kit, Fluorescein (Promega, Madinson, USA). The fragmented DNA of apoptotic cells was measured by incorporation of fluorescein-12-dUTP at the 3'-OH ends of DNA using the enzyme Terminal deoxynucleotidyl Transferase (TdT), which forms a polymeric tail using the principle of the TUNEL assay [20]. The fluorescein-12-dUTP nick end-labeled DNA was visualized directly by fluorescence microscopy. Cells were defined as apoptotic if the whole nuclear area of the cell was stained fluorescent. Apoptosis was expressed as number of apoptotic cells in ten fields at 400× magnification using a fluorescence microscope with a standard fluorescein filter.

Statistical analyses

Results are expressed as means ±standard deviation (SD). the Student's T-test was used to asses the statistical significance of the differences between groups and the non-treatment control.

RESULTS

Histological characteristics of tumor development in mice fed with yogurt either before or after tumor induction

In the yogurt-DMH group, changes in the large intestine were followed from the first month after carcinogen administration and compared with the non-treatment control group. Small multifocal infiltrates in the lamina propria were observed after the first month. In the fourth month they were bigger and the infiltrative cells occupied all the lamina propria and destroyed the Lieberkühn's glands (Figure 1A). A great vascular congestion was seen in the fifth and sixth months and the basal membrane was broken and metaplasia appeared with large cubic and plane cells. Tumor tissue with the same characteristics as the DMH group was observed in the seventh month.

The group of mice in which yogurt was administered after tumor induction showed, in the first and second months, nodular infiltrates circumscribed in the lamina propria. Small diffuse infiltrates were observed in the lamina propria in the third and fourth months after DMH injection, when the mice were fed cyclically with yogurt. The glandular structure was conserved during the whole experiment. Vascular congestion was not observed. At the end of the study (months five and six), a cellular increase was seen in the lamina propria (Figure 1B).

Study of IFNγ, TNFα and IL-10 cytokines in the large intestines of the yogurt-DMH group

IFNγ, TNFα and IL-10 showed significant increases ($P<0.001$) in their levels in all months compared with the non-treatment control group (Figure 2). The values for these cytokines were also increased in the yogurt-DMH-yogurt group and were already shown by Perdigón et al. [18].

Measurement of IL-4$^+$ cells

IL-4 was significantly increased ($P<0.001$) in the yogurt-DMH-yogurt group in the second month

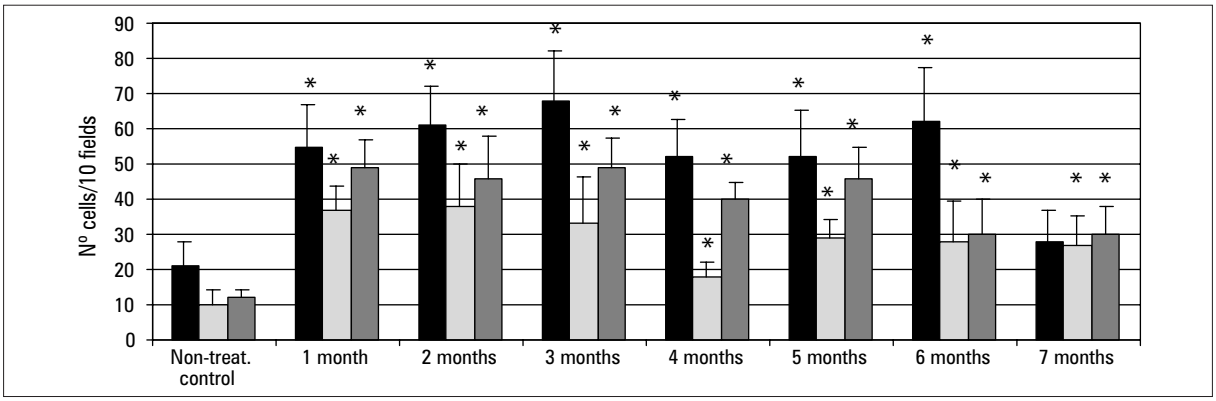

Figure 2. Effect of yogurt feeding before DMH injection on cytokines in the large intestine. Positive cells for each cytokine,IFNγ (black bars), TNFα (white bars) or IL-10 (grey bars) were determined on histological sections from large intestine of the non-treatment control and yogurt-DMH groups by indirect immunofluorescence assay. Values are means for n=5 ±SD. Significant differences were calculated in comparison with the non-treatment control group *=P<0.001.

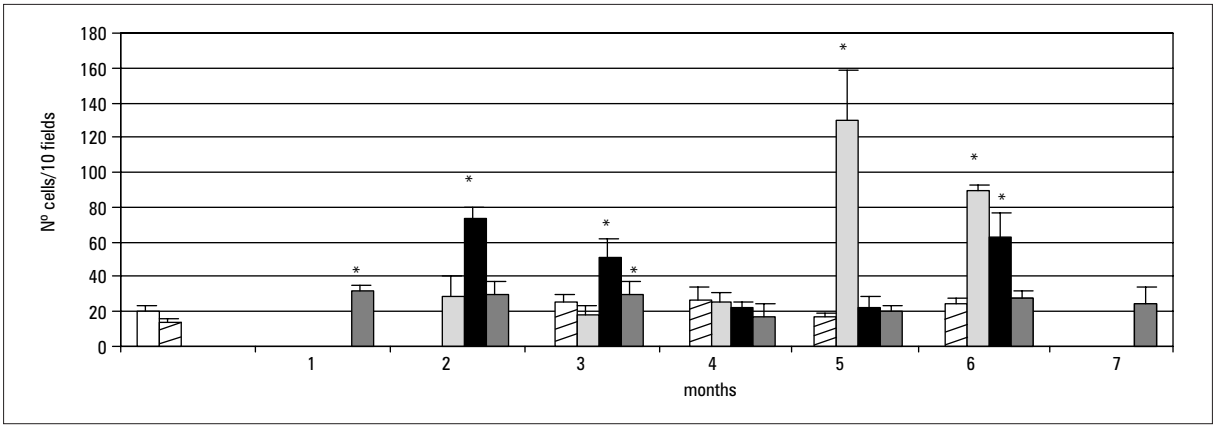

Figure 3. IL-4 positive cells in the large intestine for the different test mouse groups. Positive cells for IL-4 were counted in histological sections from the large intestine of the DMH (grey clear bars), yogurt-DMH-yogurt (black bars), yogurt-DMH (grey bars), yogurt (lined bars) and non-treatment control group. Values are means for n = 5 ±SD. Significant difference were calculated in comparison with the control group. *=P<0.001.

(74±6). At the end of the study (month six), this cytokine increased again in comparison with the non-treatment control group (63±14 and 20±3, respectively). The mice of the tumor control group showed significantly high IL-4 values in regard to the control in the fifth and sixth months (170±29 and 89±4). The mice fed long term with yogurt did not shown IL-4 increase during the treatment. In the yogurt-DMH group, IL-4 was significantly enhanced (P<0.001) only in the first month after DMH injection began (32±7), then the values decreased until the fourth month and increased again in the sixth and seventh months (28±9 and 25±9). Figure 3 shows these results.

Detection of IFNγ and TNFα in cells from the large intestine nodules

The mice of the yogurt-DMH-yogurt group showed a significant increase (P<0.01) in TNFα at months two, three and four, when the total cells were analyzed. These values were compared with the animals of the tumor control group for each period (Table 1). In the sixth month, TNFα levels increased again in the yogurt-DMH-yogurt group (263±14), but it was not possible compare them with DMH control, which developed tumor. Both adherent and non-adherent cells showed significant TNFα production.

When total cells were observed, IFNγ was enhanced for all months in the yogurt-DMH-yogurt group compared with the tumor control group. IFNγ was detected in adherent as well as non-adherent cells (Table 1).

Measurement of TNFα and IFNγ in cells isolated of Peyer's patches

TNFα was significantly increased (P<0.01) in mice fed with yogurt compared with the non-treatment control group after ten days of feeding. In the other analyzed periods of time, the TNFα-positive cell numbers were similar to the control. In the cells from the control group one could observe that adherent and non-adherent cells were positive for TNFα. Table 2 shows these results.

Table 1. Cytokine positive cells in the isolated cells of large intestine nodule.

Groupof treatment	Period of treatment	Cytokines					
		TNFα			IFNγ		
		Total cells	NA cells	A cells	Total cells	NA cells	A cells
DMH	2 months	53±14	ND	ND	90±13	ND	ND
	3 months	80±15	26±7	53±13	197±18	22±4	29±4
	4 months	19±9	18±3	53±14	14±3	15±3	63±8
	5 months	95±13	190±50	32±1	34±10	151±4	32±1
	6 months	ND	ND	ND	ND	ND	ND
Y-DMH-Y	2 months	157±18*	ND	ND	161±20	48±4	ND
	3 months	632±54*	66±4	66±4	224±20	17±2	62±8*
	4 months	142±5*	73±5*	73±5	52±2*	104±4*	29±2
	5 months	112±2	71±7	71±7*	120±4*	288±11*	46±1
	6 months	263±14	55±4	55±4	219±12	224±5	61±10

Results are expressed for the total and non-adherent cells as means ±standard deviation (SD) of all cytokine-positive cells in one well (cells/well). For adherent cells the results are the means ±SD of cytokine-positive cells each 100 counted cells (cells/100).

* significant differences of yogurt-DMH-yogurt (Y-DMH-Y) group compared with the DMH group for each month.

P <0.01. NA – non-adherent; A – adherent; ND – not determined

Table 2. Cytokine-positive cells in isolated cells of Peyer's patches.

Groupof treatment	Period of yogurt feeding	Cytokines					
		TNFα			IFNγ		
		Total cells	NA cells	A cells	Total cells	NA cells	A cells
Yogurt	4 months	24±5	7±1	74±5*	351±14*	17±1	42±7*
	5 months	22±9	52±5*	3±1	32±1	49±15*	14±2
	6 months	16±3	52±17	ND	13±2	28±10*	ND
Basal yogurt	10 days	164±16*	13±2	20±6 *	105±11*	32±1*	63±9*
Non-treatment control		20±6	18±2	8±4	19±2	8±4	6±2

Results are expressed for total and non-adherent cells as means ±SD of cytokine-positive cells in one well (cells / well). For adherent cells the results are the means ±SD of cytokine-positive cells each 100 counted cells (cells/100).

* significant differences between the yogurt group and non-treatment control.

P <0.01. NA – Non-adherent; A – Adherent; ND – Not determined

IFNγ was significantly increased (P<0.01) after ten days of yogurt feeding compared with the control. This enhancement was observed in both adherent and non-adherent cells. At month four, IFNγ continued to be enhanced in total cells, with an increase in the cytokine production from adherent cells compared with the control. See Table 2.

Measurement of CD25$^+$ and CD4$^+$ cells

CD25$^+$ cells were increased in the third month in the yogurt-DMH group and in the two analyzed months for the yogurt-DMH-yogurt group. CD4$^+$ cells only showed an increase for the yogurt-DMH group in the third month. In the other samples, the number of CD4$^+$ cells was similar to that of the non-treatment control. See Table 3.

Apoptosis measurement

The yogurt-DMH group showed an increase (P<0.01) in the number of apoptotic cells for the first months compared with the control. The number of apoptotic cells decreased in months five and six. In mice from the DMH-yogurt group, a significant increase was observed in the number of apoptotic cells (P<0.01) compared with the non-treatment group from the second month, when yogurt was added to their diet. Only in the fifth month did the values decrease, and they increased again in the sixth month. See Table 4.

Table 3. Effect of tumor induction and yogurt feeding on regulatory CD4/CD25 + T cells.

Groups of treatment	Period of treatment	CD25+cells	CD4+cells	IL-10+cells
Y-DMH	3 months	45±11*	33±11*	49±8*
	7 months	11±2	20±5	30±8*
Y-DMH-Y	4 months	19±5*	15±4	88±12*
	6 months	25±7*	14±3	40±11*
Non-treatment control		11±4	20±3	12±2

The values are expressed as means ±SD of the number of positive cells (fluorescent cells) counted in ten fields of vision at 1000X magnification (cells/10 fields).

* significant differences of yogurt-DMH-yogurt (Y-DMH-Y) or yogurt-DMH (Y-DMH) groups compared with non-treatment control. P <0.001

Discussion

In a previous study it was reported that, using an intestinal tumor model, mice fed with yogurt before and

Table 4. Apoptosis induction by yogurt feeding before and after of DMH injections.

Groups of treatment	Time of treatment	No. apoptotic cells/10 fields
Yogurt-DMH	1 month	52±12*
	2 months	60±14*
	3 months	82±16*
	4 months	55±11*
	5 months	13±2
	6 months	6±2
DMH-yogurt	1 month	24±6
	2 months	82±13*
	3 months	61±19*
	4 months	177±8*
	5 months	19±2
	6 months	33±6
Basal yogurt	10 days	34±6
Non-treatment control		25±3

The results are expressed as means ±SD of the number of apoptotic cells counted in ten fields of vision at 400× magnification (cells /10 fields).
* significant differences between the sample of test groups and the non-treatment control group. $P < 0.001$

after DMH injections (the yogurt-DMH-yogurt group) did not develop colon cancer throughout the period assayed (six months) in contrast to a tumor control group, which showed intestinal tumor in the fifth or sixth month [8]. In order to find out at which stage of the tumor process yogurt acted (the initiation, promotion, or progression of tumor growth), two additional experimental groups were studied to determine whether prior feeding with yogurt was sufficient in itself to attain the regulatory immune response observed, or whether cyclical administration of yogurt was necessary to prevent the effect of the DMH carcinogen.

Histological studies from the large intestine showed that prior yogurt feeding for 10 days before DMH injections only delayed tumor appearance; thus yogurt administration prior to DMH injections was not enough to inhibit the tumor at the initiation stage (Figure 1A). Mice fed with yogurt after tumor induction showed amelioration in the large intestine when the yogurt was added cyclically to their diet (Figure 1B), indicating that yogurt feeding can inhibit tumor promotion and progression. The behavior of this last group was similar to that of the yogurt-DMH-yogurt group previously studied [8]. In both groups, the mice not only showed a lack of tumor development, but also significant cell infiltration into the lamina propria. This last observation was also seen in the large intestines of mice fed long-term with yogurt (data not shown). The cytokine determinations allowed us to study the importance of these cells in the immune response modulation caused by yoghurt. To do this, different cytokines were analyzed in the mice of the yogurt-DMH group for comparison with the yogurt-DMH-yogurt group in which the cytokines had previously been analyzed. It was reported that TNFα and IFNγ were increased in cells of the large intestine of the tumor control mice and of mice from the yogurt-DMH-yogurt group, and that yogurt feeding itself also produced high levels of these cytokines [14]. In this study, IFNγ and TNFα were investigated in order to understand if the delay in tumor appearance is mediated by these cytokines which are stimulated by yoghurt, but we could not assign any role to them because they were increased in all the periods assayed for the mice (Figure 2).

IL-10 and IL-4 were evaluated due to their immunoregulatory capacity: they play a significant role in controlling cell growth and modulating the immune response. IL-4 is mainly secreted by stimulated CD4+ T helper, mast, and basophil cells and exhibits anti-inflammatory properties which inhibit the production of several proinflammatory cytokines such as IL-1, IL-6, IL-8, IFNγ and TNFα (21). IL-10 is important as a regulator in the intestine, and the number of IL-10+ cells increased significantly in all the mice groups of our experimental tumor model [14].

The number of IL-4$^+$ cells decreased throughout the duration and increased again in the end of the experiment, while the increase in IL-10$^+$ cells persisted in the large intestine cells after yogurt feeding was stopped.

IL-4 was also determined in the experimental groups studied by Perdigón et al. [14] because it is an antagonist of IFNγ and in previous works with those mice an increase in the IFNγ values was observed in all the groups, but only the animals that presented tumor had high levels of iNOS-positive cells [18], showing the anti-inflammatory properties of yogurt. In the present study, the number of IL-4$^+$ cells increased at the beginning of the experiment in the yogurt-DMH-yogurt group; IL-4 probably exerts control over the inflammation, which helps in the non-development of the tumor. Afterwards, the number of IL-4$^+$ cells was similar to that obtained in the non-treatment control group (Figure 3). It is important to note that yogurt feeding by itself did not increase IL-4$^+$ cells, although it increased IL-10$^+$ cells in the large intestine [14]. On other hand, IL-4$^+$ cells only increased at the end of the experiment (six months) in the tumor control group, at which time the mice presented significant lesions in the large intestine and development of tumor. In this group it is possible that the great alterations produced in the intestine together with the high levels of cytokines such as IFNγ and TNFα increased the IL-10 levels to regulate the immune response [14]. This could not by itself control the inflammatory response induced by the carcinogen, so IL-4 regulatory cytokine was released. However, at this stage of tumor evolution (fourth and fifth months), tumor growth could be not reverted.

The cells from nodules in the large intestine showed increases in their number when the mice were injected with DMH and they are important in the study of immune responses in the DMH as well as the yogurt-DMH-yogurt groups. Belardelli and Ferrantini [22] studied the possible role of cytokines produced by cells of the innate defense system (such as TNFα and IFNγ) in the protective antitumor immunity. In our model, the increased CD4$^+$ T lymphocytes reported in mice treated with DMH and fed with yogurt could be one of

the cells producing IFNγ, IL-4, and IL-10 [8]. TNFα production was determined because it could also be related to the enhancement in cellular apoptosis observed when mice injected with DMH received yogurt as a diet supplement [23].

It was demonstrated that in the yogurt-DMH-yogurt group, yogurt feeding effectively increased TNFα$^+$ cells compared with the tumor control group. These results showed that in the presence of the carcinogen, mononuclear cells in the nodules produced TNFα, but yogurt increased their cell activity with an enhancement of this cytokine. It is known that the cells producing of TNFα belong to non-adherent (fibroblast, mast cells, and some T and natural killer cells) as well as adherent (macrophages/monocytes) cells [21]. Here it was shown that although there were non-adherent cells producing TNFα, the adherent cells were more important producers of this cytokine (Table 1). IFNγ also increased with yogurt supplement (Table 1). This cytokine is produced principally by cells belonging to the non-adherent populations, such as T cells and natural killer cells [21]. However, IFNγ can be released by other cells, such as macrophages and dendritic cells, which are considered adherent cells [24]. We observed that, in addition to non-adherent cells, adherent cells also possessed IFNγ (nearly 50%). All these observations show that yogurt stimulates the infiltrative cells (adherent and non-adherent), which increased the cytokine production necessary for tumor resolution.

Considering these previous results, we analyzed the effect of yogurt feeding on TNFα and IFNγ cytokine production from cells of Peyer's patches. We used Peyer's patches because this is an inductor site of the intestinal mucosal immune response, and in this group of animals it was not possible to isolate immune cells from the large intestine nodules due to the small size. Our results showed that yogurt only stimulates the cytokine production by Peyer's patch cells at the beginning of yogurt supplementation, after which a basal cytokine level is maintained. This suggests that yogurt could modulate the immune response: it stimulates cytokine production when this is required (in presence of a carcinogen, for example) or induces their down-regulation to avoid exacerbation of the immune response. This last effect would be mainly through IL-10, which showed increases in the tissue of mice fed with yogurt during all the periods assayed.

CD4$^+$ T lymphocytes were enhanced in mice fed with yogurt before and after DMH injections [8]. There is evidence that CD4$^+$ T cells which express the CD25 marker play a critical role in immune regulation through IL-10 production [25], but there are discrepancies regarding the regulatory activity and the CD25 marker. Curotto de Lafaille and Lafaille [26] reported that in many situations CD4$^+$/CD25$^-$ T cells are as effective as CD4$^+$/CD25$^+$ T cells in controlling T cell-mediated diseases. We analyzed CD25$^+$ cells from the large intestine from different treatment periods where the number of IL-10$^{(+)}$ cells was highest compared with samples where the number of IL-10$^{(+)}$ cells was lowest. We saw that the CD25 marker was only increased in some samples (Table 3) and did not show any relation with the CD4 marker and IL-10 production. In this way, the CD4$^+$/CD25$^+$ T regulatory cells are not responsible for the increased levels of IL10 observed in our tumor model.

On other hand, Rachid et al.[23] reported that the increase in cell apoptosis is one of the mechanisms involved in the tumor inhibition by yogurt. Since cytokines such as TNFα could be involved in certain apoptotic pathways [27], one of the mechanisms for the control of tumor growth, and since we determined an enhancement of this cytokine, we studied apoptosis induction in the two groups of mice that were analyzed histologically. It was confirmed that yogurt supplementation stimulated apoptosis in the large intestine. Mice fed with yogurt before DMH injections only increased apoptosis until the fourth month (Table 4). This observation and the enhancement of regulatory cytokines induced by prior yogurt diet could explain the delay in tumor appearance in this group of mice. In the group of mice fed cyclically with yogurt after tumor induction, an increase in the number of apoptotic cells was observed during the whole experiment after yogurt was given to the mice, similar to the yogurt-DMH-yogurt group as reported by Rachid et al. [23].

CONCLUSIONS

We demonstrated that yogurt exerts its antitumor activity through stimulation of the immune system; such activation was observed in Peyer's patch cells as well as in the large intestine. When the carcinogen was present, yogurt increased cytokine production in the large intestine (lamina propria and nodules), mainly caused by cells from the adherent population.

Even though yogurt feeding before the carcinogen injections showed rapid regulatory cytokine release and apoptosis stimulation in the large intestine, this only-yogurt supplementation was incapable of inhibiting tumor appearance and development. Yogurt must be cyclically administered after DMH to inhibit tumor growth. Our results demonstrated that yogurt exerts its antitumor activity by inhibition of tumor progression and promotion; this effect is reached through long-term and cyclical yogurt consumption.

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