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Antitumour effect of *Lactobacillus casei* CRL 431 on different experimental tumours

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Abstract

We analysed the mechanisms of tumour inhibition by *Lactobacillus casei* CRL 431 orally administered, for fibrosarcome (non intestinal tumour, NIT) and for carcinoma (intestinal tumour, IT), both induced chemically. Balb/c mice were fed with *L. casei* (1.2×10^9cel/day/mouse) for two consecutive days previous to the induction of the respective tumours. After IT induction, oral administration of *L. casei* was repeated cyclically every 5 days for 5 months. In order to determine the percentage of tumour inhibition in the NIT mice, morphometric studies were performed and TNFα levels in serum and spleen cells were determined. In the IT, macroscopic and histopathological studies were performed and the number of intestinal IgA^+ and $TNF\alpha^+$ cells was determined. We demonstrated that *L. casei* CRL 431 inhibited the growth of both tumours. In the case of NIT animals treated with *L. casei*, the high levels of serum TNFα would play an important role in the inhibition of tumour growth due to its cytolytic activity against tumour cells. In the IT, the increase in IgA^+ and $TNF\alpha^+$ cells could be involved in the inhibitory effect observed. Our results showed that the selection of the probiotic bacteria with antitumour activity would not be limited to the determination of the cytokines relevant to this activity because of the multifactorial etiology of the different cancers.

Keywords: Lactobacillus casei, *fibrosarcome, colorectal cancer, cytokines.*

Introduction

In recent years, lactic acid bacteria have attracted increasing attention throughout the world due to both their economic significance in the food industry and their role in maintaining gut health. Among these bacteria, *Lactobacillus casei*, a probiotic bacterium has been reported to possess several biological activities (Hayatsu and Hayatsu 1993, Kato et al. 1984).

Perdigón et al. (1993) demonstrated that the oral administration of *L. casei* CRL 431 activated immune mechanisms and inhibited the progression of a subcutaneously implanted fibrosarcome, the effect of these microorganisms being intimately related to their dose.

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Other lactobacilli strains showed antitumour effects not only in rodents but also in humans (Femia et al. 2002). In animal studies, the intralesional injection of lactobacilli effectively inhibited tumour growth (lung carcinoma) (Masuno et al. 1991); the oral administration of lactobacilli also prevented tumour development (colon/bladder cancer) (Aso et al. 1995).

The *L. casei* strain Shirota (LC 9018) has been reported to exhibit strong antitumour activity and to inhibit metastases in allogeneic and syngeneic mouse and guinea pig systems (Kato et al. 1981, Matsuzaki et al. 1985). One of the mechanisms of action of LC 9018 has been proved to be dependent on the increase of the host cellular immune response, such as NK and macrophage and $CD8^+$ cytotoxic activity (Yasutake et al. 1984, 1985), and on the production of cytokines such as TNFα, IFN-γ and IL-12 (Yasutake et al. 1999).

Colorectal cancer is one of the main causes of cancer morbidity and mortality in Western countries (Parker et al. 1996). The oral administration of lactic acid bacteria has proved effective to reduce DNA damage caused by chemical carcinogen, in gastric and colonic mucosa in rats. Several lactic acid bacteria have been reported as protective towards 1,2-dimethylhydrazine (DMH)-induced genotoxicity (Pool et al. 1993).

The consumption of fermented dairy products has been proposed in order to provide protection against carcinogenesis. Perdigón et al. (1998) demonstrated that yogurt inhibited an experimental intestinal tumour. They showed that one of mechanisms by which yogurt inhibits the establishment and the growth of the tumour could be through a down regulation in the inflammatory immune response. This regulation could be due to the increase in the IgA cells, IL-10, cellular apoptosis and to the diminution of iNOS enzyme (De Moreno de Leblanc et al. 2004).

In a previous work (Perdigón et al. 1993), we demonstrated the adjuvant effect of *L. casei* CRL 431 on the immune system. The present investigation was carried out in order to determine the preventive effect of the oral administration of *L. casei* CRL 431 on the inhibition of an intestinal tumour (carcinoma) induced with DMH and of a fibrosarcome induced with methylcholanthrene (Meth A). The role played by IgA cells and TNFα in the preventive effect on both tumours is also analysed.

Material and methods

Animals

BALB/c mice, each weighing 25–30g, were obtained from a random-bred closed colony kept in our Microbiology Department. Each experimental group consisted of 20–30 mice. For each assay 4–5 mice were used.

Microorganisms

The strain used was *L. casei* CRL 431 from CERELA. *L. casei* was cultured in Rogosa medium for 24 h at 37°C. After growth, the microorganisms were collected by centrifugation, washed with saline solution (0.9% NaCl) and suspended in 5 ml of sterile non-fat milk (NFM) at 10% before use.

Feeding procedure and fibrosarcome induction

Prior to inoculation with tumour cells, mice were fed for two consecutive days with 1.2×10^9 CFU/day/mouse, the optimal dose determined in a previous paper (Perdigón et al. 1993). The viable culture was suspended in 5 ml of sterile NFM at 10% and administered at a 20% (v/v) concentration in the drinking water. The control group was given sterile NFM at 10% in the drinking water. All animals were fed *ad libitum* with a standard diet.

The non intestinal tumour used was a fibrosarcome induced by the subcutaneous implantation of Meth A crystals maintained by *in vivo* serial passages. Transplants were made by the subcutaneous inoculation of 5×10^5 viable tumour cells into the left flank of *L. casei*-treated mice at the end of each feeding period and into the control mice without *L. casei* treatment.

Measurement of fibrosarcome tumour size

Tumour growth was evaluated by calliper measurement of tumour length, tumour volume being determined by the formula of Attia and Weiss (1966):

$$V = 0.5 \times d^2 \times D$$

where V stands for tumour volume (cm^3), d for shorter diameter and D for longer diameter.

The inhibition rate for tumour growth was calculated by the following formula:

$$\text{Inhibition rate } \% = 1 - \frac{\text{Mean tumour weight of treated mice}}{\text{Mean tumour weight of control mice}} \times 100.$$

Determination of TNFα

TNFα was tested by ELISA in serum and in spleen cells. The samples were obtained from three groups of mice: (1) normal control, (2) tumour control group, without pre-treatment with lactic acid bacteria, and (3) animals pre-treated with *L. casei* for two consecutive days before tumour cells inoculation.

The serum TNFα assay was performed by the quantitative sandwich enzyme immunoassay technique with a kit from Quantikine M murine, R&D Systems Inc. (Minneapolis, USA).

TNFα present in isolated spleen cells was measured by an immunohistochemical method as follows. The spleen of each mouse was removed, washed and placed in Hank's balanced saline solution (HBSS) with fetal bovine serum (FBS) added. The cells were separated by stirring with a magnetic agitator. After addition of a haemolytic solution, the centrifugation was repeated and the supernatant was discarded. The cells were suspended at a final concentration of 4×10^6 cells/ml in HBSS.

The cells in a volume of 20 µl were placed on glass slides and fixed with 10% formalin (ICC fixation buffer, PharMingen). Then they 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 cytokine (TNFα) polyclonal antibodies (diluted in 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 Laboratories) was added to cells which were incubated with 3,3 diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA). The results were expressed as a percentage of positive cells (counted at 100 ×) using a light microscope.

Intestinal tumour and feeding procedure

The intestinal tumour was induced with the carcinogen DMH (Sigma). Each mouse received subcutaneously 20 mg of DMH/kg body weight in 0.1 ml of a saline solution containing 1.5 g/l of EDTA, pH 6.4, weekly for 10 weeks. Tumour incidence was $70 \pm 5\%$. Only animals that developed tumours were used in the experiments (tumour control or DMH group).

The animals were separated into three experimental groups:

1. DMH group: Mice were treated only with 1,2 dimethylhydrazine;
2. *L. casei*-DMH-*L. casei* group: Mice were given *L. casei* (1.2×10^9 CFU/day/mouse) for two consecutive days previous to the first inoculation with DMH. On week 10 after tumour induction, *L. casei* administration was repeated cyclically. Mice were given *L. casei* for 2 consecutive days every 5 days for 10 weeks post tumour induction;
3. *L. casei* group: Mice were given *L. casei* for 2 consecutive days, every 5 days for 10 weeks.

Histological studies for the intestinal tumour

Mice from the three groups above 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 according to Sainte Marie's technique (Sainte-Marie 1962). Serial paraffin sections of 4 mm were cut; some were stained with haematoxylin-eosin for light microscopy examination and others were used for the immunofluorescence assay.

Immunofluorescence assay for IgA secreting cells and TNFα cytokine in the large intestine

The number of IgA secreting cells was measured on months 1, 2, 3, 4 and 5 after tumour induction with a direct immunofluorescence assay, using α-chain monospecific antibody conjugated with fluorescein isothiocyanate, FITC (Sigma).

$TNF\alpha^+$ cytokine was tested throughout the same period after tumour induction as IgA, using a polyclonal rabbit antimouse TNFα antiserum (Peprotech, Inc., Rocky Hill, NJ, USA) and, as second antibody, a goat anti-rabbit antibody conjugated with FITC (Jackson Immuno Research Labs Inc., West Grove, USA). Results were expressed in both determinations counting fluorescent cells in 30 fields at 100 × of vision.

Statistical analyses

Results were expressed as the mean ± standard deviation (SD). Student's *t*-test was used to assess the statistical significance of the differences between the test group and the non-treated control.

Results

Effect of the oral administration of L. casei *on the growth of a fibrosarcome*

In the tumour control group, on days 10–13 after tumour induction, we observed that the tumour had reached a volume of 0.3 ± 0.01 cm^3 and was perfectly palpable at this stage in $70 \pm 5\%$ of the animals ($n = 25$). On days 20–25 the tumour volume in these animals was about 2 ± 0.1 cm^3. Mice died between 30 and 40 days after tumour inoculation, mortality being recorded up to day 40. At this time the tumour volume was about 3.2 ± 0.1 cm^3 (see Table I).

A total of $54 \pm 5\%$ ($n = 25$) of the mice pre-treated with *L. casei* for two consecutive days did not present tumour development, while in the remaining $46 \pm 4\%$ ($n = 25$), a delay of 30 days in the development of the tumour (vol: 1.2 ± 0.05 cm^3) was observed. After that time, tumours grew at about the same rate as those observed in the tumour control (see Table I).

Influence of previous feeding with L. casei *on TNFα levels*

Results showed that feeding with *L. casei* before the inoculation of the tumour cells produced a significant increase in the levels of TNFα in blood serum (60 ± 5 pg/ml, Tumour Control (TC): 20 ± 5 pg/ml; $p < 0.001$) as well as in the spleen cells ($170 \pm 8^+$ cells/100 fields; TC: $93 \pm 6^+$ cells/10 fields; $p < 0.01$) on day 10 after inoculation. This increase was more evident in blood sera than in the spleen cells (see Figures 1 and 2). In the case of untreated animals (TC), the values were similar to the normal control (NC) in both methodologies used to evaluate TNFα (NC: serum TNFα: 17 ± 4 pg/ml, TNFα$^+$ spleen cells: $93 \pm 5^+$ cells/10 fields).

Macroscopic and histological characteristics of the intestinal tumour

In animals fed for 2 days with *L. casei*, injected with DMH and given *L. casei* cyclically every 5 days for 5 months, the presence of a few small (1–5 mm diam) non vascularized polyps was observed between the 2nd and the 5th month.

Table I. Preventive effect of *L. casei* orally administered on the inhibition of a fibrosarcome.

Groups	No. of animals w/tumour/total	% tumour growth	% tumour growth inhibition
Tumour control	17/25	70	30
Treated w/*L. casei*	14/25	0	100
	11/25*	100	0

*Tumour growth was delayed for 30 days. Animals were treated for 2 days with *L. casei*; after that they were subcutaneously injected with tumour cells. The untreated control animals developed tumour in 70% of the mice. Tumour growth was inhibited in 54% of the animals given *L. casei*. In the remaining 46%, tumour growth was delayed for 30 days.

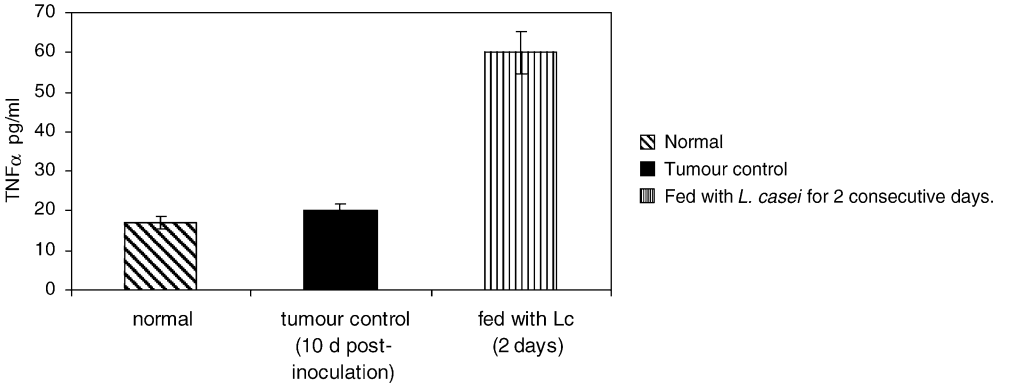

Figure 1. Effect of previous feeding with *L. casei* on serum TNFα levels in a fibrosarcome experimental tumour. Animals were treated with *L. casei* for 2 consecutive days at a concentration of 1.2×10^9cell/day/mouse. After that they were injected with 5×10^5 fibrosarcome cells. TNFα was measured in the serum of mice 10 days post-inoculation of tumour cells. Values are the mean of $n \pm$SD ($n = 5$) independent determinations.

The histological studies revealed scarce infiltration of mononuclear cells (see Figure 3a).

In the DMH control group, from month 2 onwards, we observed the presence of polyps that grew progressively up to 5–8 mm in size. On month 5, there was tumour development in $70 \pm 4\%$ of the mice. In the 2nd month, the histological analysis revealed a nodular infiltration in the large intestine, with a great infiltration of mononuclear cells that extended within the glands from the base up to the apical zone. On the 5th month, an adenocarcinoma developed with multilayered tumour cell disruption. The connective tissue stromas were spread throughout the tumour and infiltrated with mononuclear cells in addition to connective tissue (see Figure 3b).

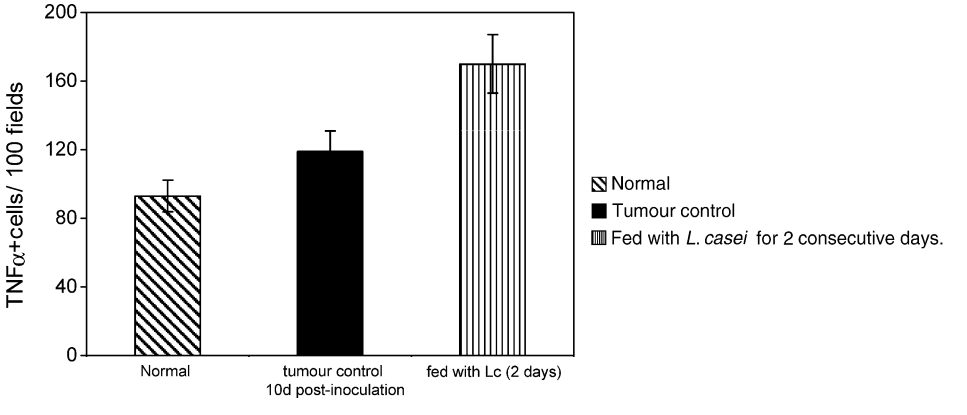

Figure 2. Influence of previous feeding with *L. casei* on TNFα$^+$ spleen cells in a fibrosarcome tumour induced with Meth-A. Mice were fed with *L. casei* for 2 days. Then, they were inoculated with tumour cells in the same way as the untreated control animals. On day 10 post-inoculation, the spleen was removed and TNFα cytokine was determined in the spleen cells. Values are the mean of $n \pm$SD ($n = 5$) independent determinations.

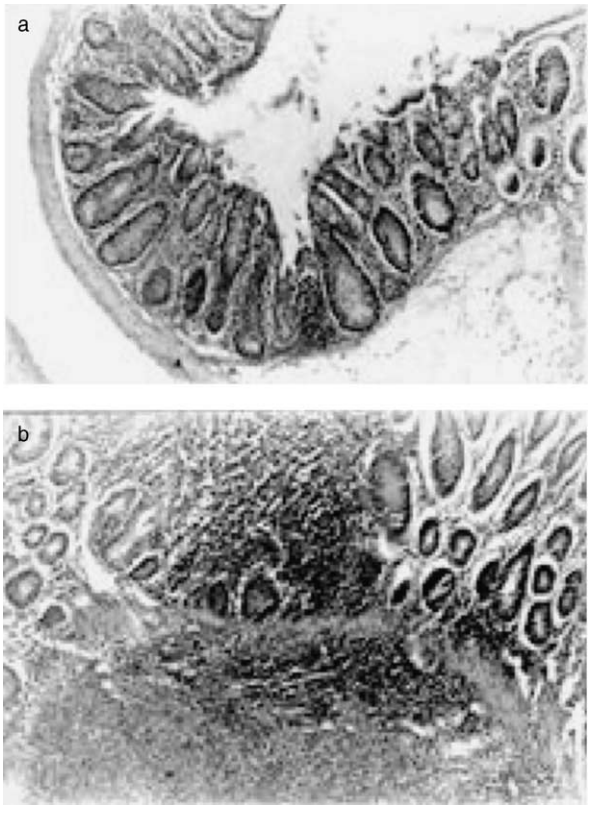

Figure 3. Effect of *L. casei* on the inhibition of a DMH induced tumour. Animals were fed with *L. casei* for 2 days previous to DMH injection. On the 10th week after tumour induction, feeding with *L. casei* for 2 days was repeated cyclically every 5 days for 10 weeks. Light micrograph (magnification 40 ×) of haematoxylin-eosin stained sections from the large intestine from the *L. casei*-DMH-*L. casei* group (a) and from the DMH control group (b). On week 10, treated animals only showed immune cell infiltration in the lamina propria of the large intestine. Untreated animals showed severe histological alteration with polyps development.

Number of IgA secreting cells in the large intestine

We observed a marked increase in the number of IgA secreting cells ($p > 0.001$) in the *L. casei*-DMH-*L. casei* group (127 ± 6 IgA^+ cells/10 fields, 1st month) in relation to both DMH group (80 ± 5 IgA^+ cells/10 f, 1st month) and *L. casei* group (100 ± 6 IgA^+ cells/10 f, 1st month). This increase was more evident from months 2–5 in the *L. casei*-DMH-*L. casei* group (223 ± 10 IgA^+ cells/10 f, 5th month) in relation to the DMH group (77 ± 6 IgA^+ cells/10 f, 5th month) and the *L. casei* group (80 ± 6 IgA^+ cells/10 f, 5th month). These results are shown in Figure 4.

Number of TNFα^+ cells in the large intestine

$TNF\alpha^+$ cells ($p < 0.001$) showed a significant increase only in the group treated with the carcinogen in all the periods assayed: *L. casei*-DMH-*L. casei* group, 75 ± 11 $TNF\alpha^+$ cells/10f, 3rd month; DMH group, 250 ± 15 $TNF\alpha^+$ cells/10 f, 3rd month. This increase was also significant ($p < 0.001$) until the 5th month. In the same period, the *L. casei* group (76 ± 5 $TNF\alpha^+$ cells/10 f, 5th month) showed a

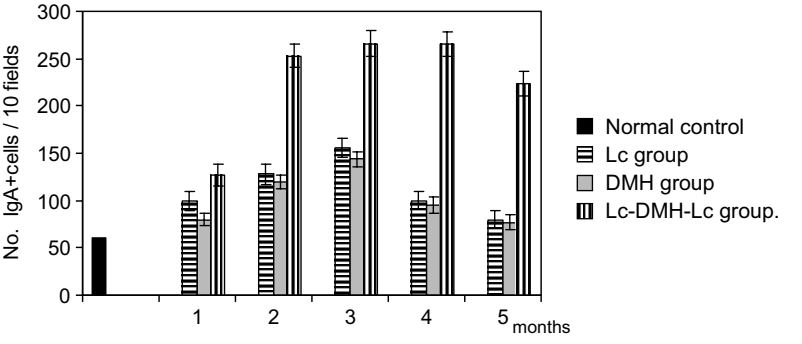

Figure 4. Effect of long term administration of *L. casei* on IgA-secreting cells in the large intestine in a carcinoma experimental tumour. The number of IgA-secreting cells was determined in the lamina propria of the large intestine by direct immunofluorescence. Values are the mean of $n \pm$SD ($n = 5$) independent determinations.

significant enhancement in TNFα^+ cells values ($p > 0.01$) in relation to normal controls (15 ± 4 TNFα^+ cells/10 f). These results are shown in Figure 5.

Discussion

In previous works Perdigón et al. (1993, 1995) suggested that the preventive effect of the oral administration of *L. casei* on the inhibition of the growth of a fibrosarcome would be mediated by the release of seric factors. These factors are able to mediate the lyses of tumour cells as demonstrated by *in vitro* cytotoxic assays using peritoneal macrophages as effector cells. The antitumour activity of macrophages may involve direct contact with the tumour cells and the secretion of cytokines such as TNFα. This cytokine exhibits cytostatic and cytocidal effects on several tumour cells lines *in vitro* (Urban et al. 1986, Kato 2000).

Kato et al. (2000) demonstrated that macrophage activation by *L. casei* is an important step in the acquisition of T-cell-mediated antitumour immunity. They suggested that T cells contribute to the development of a specific antitumour activity by direct cytotoxicity and macrophages activated by *L. casei* would contribute directly to the killing of tumour cells thought cytokine release.

Our results show that the mice pre-treated with *L. casei* and then injected with fibrosarcome cells, exhibited a significant increase in the levels of TNFα both in serum and in spleen cells (see Figures 1 and 2). These results agree with those of Yasutake et al. (1999), who reported that *L. casei* 9018 exhibited strong antitumour activity in a murine model of malignant pleurisy induced with Meth A cells. These authors clearly demonstrated that TNFα was one of the main effector molecules in the antitumour activity of *L. casei* 9018.

TNFα is a cytokine with a dual role that depends on the levels produced. It can either be proinflammatory or induce a regulatory effect to mediate cellular apoptosis (Feghali and Wright 1997, Seller and Fisher 1999). We demonstrated that the high levels of TNFα observed in the fibrosarcome model were found in $54 \pm 5\%$ of the mice pre-treated with *L. casei,* which did not show tumour development (see Table I). This finding suggests that TNFα would be involved in a protective effect rather than in an inflammatory one. Kato et al. (1994) also reported the antitumour activities of *L. casei*

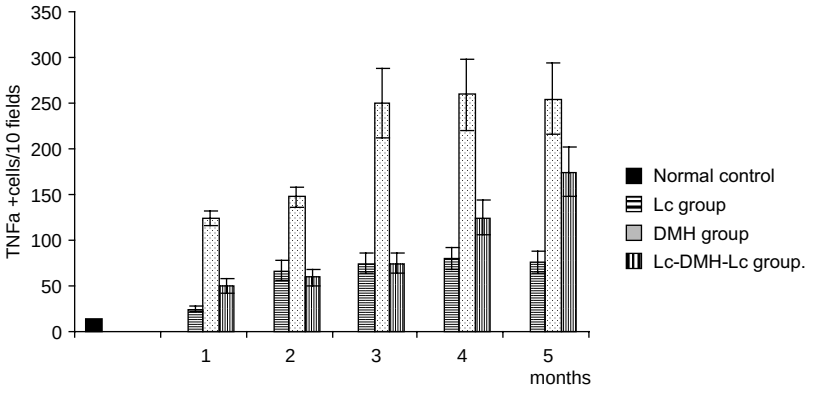

Figure 5. Effect of *L. casei* on the number of TNFα proinflammatory cytokines in an induced intestinal tumour. The proinflammatory cytokine (TNF-α) produced in the large intestine by mice injected with the carcinogen DMH and treated with *L. casei* orally administered was determined by direct immunofluorescence every month until month 5. Values are the mean of n ± SD ($n = 5$) independent determinations.

9018 and suggested that tumour suppression may have resulted from the eradication of cancer cells via augmentation of immune surveillance.

It has been demonstrated that the increase in the number of IgA-secreting cells in the large intestine of mice given yogurt can modulate the inflammatory immune response (Perdigón et al. 1998), since IgA is considered an immune barrier in colonic neoplasia (Issacson 1982). When we analysed the effect of long-term *L. casei* administration, we noticed an increase of IgA-secreting cells in the lamina propria of the large intestine, in all the periods assayed (see Figure 4). This increase in IgA induced by *L. casei* treatment would be involved in the anti-inflammatory effect. This speculation is based on our histological studies, which revealed no polyps in the animals fed with *L. casei* (see Figure 3a).

Probiotic bacteria can induce multiple cytokines (Perdigón et al. 2002), which play an important role in the protection against tumours. At the intestinal level, TNFα production is important in resolving inflammatory lesion, leading to apoptosis of immune cells, which prevents the persistence of the inflammatory infiltrate, as demonstrated in previous papers (Buckley et al. 2001, De Moreno de Leblanc et al. 2004). In our intestinal tumour model, we found that TNFα levels were increased in all the groups under study, suggesting that this cytokine would also be involved in the inhibition of the experimental colorectal carcinoma studied. On the basis of the high TNFα values found in the DMH group even when we did not perform the apoptosis assay, we cannot rule out other possible roles for this cytokine besides cellular apoptosis.

We demonstrated that *L. casei* given cyclically to mice delays the development of a colorectal carcinoma. This effect would be mediated by an increase in anti-inflammatory IgA and TNFα which, in turn, would mediate protective mechanisms. In the intestinal tumour, regulatory cytokines such as IL-10 or IL-4 could play a more important role than TNFα in the regulation of the inflammatory response induced by the carcinogen. The suppression of carcinogenesis by *L. casei* may involve other mechanisms such us a modification in the metabolism and the excretion of chemical carcinogens as a result of an alteration in the intestinal microflora.

These studies suggest that the mechanisms through which a certain probiotic bacterium exerts its antitumour effect would not be the same in different types of tumours. Such an effect would depend on the site of tumour development, on the type of cells involved (epithelial or connective tissue cells), and on the cytokines released as a consequence of probiotic stimulation. The behaviour of a certain probiotic strain capable of stimulating different mechanisms in the prevention or inhibition of tumour growth could be due to the antigenic complexity of its cell wall.

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