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Ability of the polysaccharide chitosan to inhibit proliferation of CD4+ lymphocytes from mucosal inductive sites, *in vitro* and *in vivo*

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Abstract

Objective: After oral administration of chitosan (a copolymer of glucosamine and N-acetylglucosamine), mesenteric lymph node (MLN) lymphocytes exhibited traits of anergy, a process coupled with inability of mature T cells to proliferate. We wondered whether biological activity of chitosan could be affecting division of lymphocytes at the mucosal inductive sites.

Materials and methods: We studied the effect of chitosan on proliferation of carboxyfluorescein diacetate-labelled MLN lymphocytes stimulated with concanavalin A *in vitro*. We assessed expression of CD25 and CD71 activation markers and pro-apoptotic molecule CD95L. Moreover, we studied the effect of chitosan *ex vivo*, in carboxyfluorescein diacetate-labelled MLN cells isolated after feeding single or repetitive doses of the polysaccharide, and we evaluated cell cycle parameters.

Results: Chitosan suppressed cell proliferation and down-modulated expression of CD25 in these MLN CD4+ cells isolated from normal rats. After *in vivo* contact, chitosan inhibited proliferation of MLN cells and reduced secretion of interferon-gamma. Furthermore, sustained feeding produced reduction in percentage of CD4+ cells in S phase of the cell cycle.

Conclusion: Here we demonstrate the ability of chitosan to suppress proliferation of CD4+ lymphocytes from mucosal inductive sites *in vivo* and *in vitro*. This effect could be relevant in modulatory activity of chitosan in the intestinal microenvironment.

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Introduction

Polysaccharides exhibit a multiplicity of effects on leukocytes. Several reports illustrate biological activity of glucose and galactose polymers on cells of the innate and adaptive immune responses. In terms of cell proliferation, polysaccharides have shown either to have stimulating or suppressing activities: purified polysaccharides from *Ganoderma lucidum* mycelium induce proliferation of human peripheral blood mononuclear cells, while *Ganoderma lucidum* spore and barley polysaccharides have weak immunostimulatory activity *in vitro* (1). Acidic polysaccharides isolated from *Phellinus linteus* stimulate proliferation of T lymphocytes and inhibit tumour growth and metastasis (2), while other polysaccharides isolated from *Phellinus* spp. (*P. linteus*, *P. baumii* and *P. gilvus*) stimulate proliferation of murine splenocytes (3).

Chitin is a polymer of β(1,4)-N-acetylglucosamine, found in various natural products, such as exoskeletons of insects and cell walls of some fungi and micro-organisms. Chitin can be hydrolysed to chitosan, a copolymer of glucosamine and N-acetylglucosamine, with increased water solubility. Chitosan has found wide application in conventional pharmaceutical devices, such as excipient, potential adjuvant or controlled drug delivery systems. Chitosan exhibits many biological actions; it is namely, hypocholesterolaemic, antimicrobial and has wound healing properties. Low toxicity coupled with wide applicability makes it a promising candidate not only for drug delivery but also as a biologically active agent (4). When cultured with chitosan, CD8+ T lymphocytes from the spleen express CD69, an early activation marker, although is not involved in cell proliferation (5). On the other hand, dietary chitosan supplements reduce development of chemical-induced precancerous lesions in the murine colon (6)

and suppress proliferation of human gastric and colon cancer cells, by inhibiting DNA synthesis (7).

Recently, we have demonstrated that oral administration of chitosan together with protein, promotes induction of tolerance towards this antigen (8). Interestingly, the polysaccharide itself enhances anti-inflammatory conditions of the mucosal environment (9). Early after chitosan administration, we found a reduction in the cellularity of Peyer's patches as well as the internalization of CD3 complex in splenocytes, possibly reflecting the activated state that precedes anergy (8). Down-regulation of T cell receptor (TCR) expression is usually tied to inability of mature T cells to proliferate and secrete IL-2 (10). On the basis of these previous findings, we hypothesize that chitosan could be affecting the proliferative capacity of lymphocytes at the mucosal inductive sites. To evaluate this possibility, we studied the effect of chitosan on proliferation of mesenteric lymph node (MLN) lymphocytes stimulated with concanavalin A (ConA). After stimulation, lymphocytes upregulated various surface markers such as CD71 and CD25 with different kinetics: the CD71 antigen is the iron-transport protein receptor that is upregulated early on activation, reflecting the iron dependence of proliferation. The CD25 antigen, a hallmark of cell activation, upregulates later, following stimulation of TCR/CD3 complex (11). On the other hand, to limit total number of stimulated cells, activated T lymphocytes induce expression of apoptotic signalling molecules, such as Fas ligand (CD95L) (12), at the cell surface. Thus, in order to evaluate the effect of chitosan on activation status of mucosal lymphocytes, we have assessed expression of CD25 and CD71 and the pro-apoptotic molecule CD95L. Moreover, to correlate these data with our previous *in vivo* findings, we have evaluated the proliferative activity of the MLN lymphocytes isolated after single or repeated feedings of chitosan. Herein, we demonstrate the ability of chitosan to suppress proliferation of lymphocytes and to inhibit the S phase of the cell cycle.

Materials and methods

Animals

In this study, we used 8- to 10 week-old female Wistar rats weighing 180–230 g. Animals were housed and cared for at the Animal Resource Facilities, Department of Clinical Biochemistry, National University of Cordoba, in accordance with institutional guidelines.

Isolation of MLN lymphocytes and feeding

MLN lymphocytes were obtained from normal rats, and single-cell suspensions were prepared in Roswell Park Memorial Institute culture medium (RPMI) supplemented with gentamicin, heparin and 10% foetal calf serum (FCS) by mechanical dispersion following standard procedures (8). For *ex vivo* studies, we used 85% deacetylated low molecular weight chitosan (~80 kDa; Sigma Aldrich, Milwaukee, WI, USA) prepared as previously described (13,14). During afternoons we fed rats a final volume of 200 μl 0.1 M acetic acid (diluent group) or acetic acid containing 1–5 mg chitosan. Sixteen hours later, we removed MLNs and prepared single-cell suspensions according to standard procedures as described above. When required, rats received five doses of either diluent or 1–5 mg chitosan every other day, and the day after the last feed we removed MLNs.

Cell proliferation and cytokine production

MLN lymphocytes (5×10^6 cells) were labelled in the dark with a 1 : 1000 dilution of 5 μM succinimidyl ester of carboxyfluorescein diacetate (CFSE; Molecular Probes, Eugene, OR, USA) for 5 min at room temperature, as previously described (15). After staining, cells were washed three times in cold 10% FCS–phosphate-buffered saline (PBS), resuspended in complete medium (RPMI medium supplemented with gentamicin, heparin and 10% FCS) and plated at 1×10^6 cells/well in 24-well plates with or without 2.5 μg/ml ConA (Sigma-Aldrich), and were cultured for 3–5 days. In some experiments, cells were cultured with 1–100 μg/ml chitosan for 24 h, washed extensively and stimulated with ConA or treated for 24 h with ConA, washed and cultured with chitosan for 3 days. At indicated times cells were harvested, stained with Cy-Chrome (PE-Cy5)-mouse anti-rat CD4 antibody (BD Pharmingen, San Diego, CA, USA) and analysed by flow cytometry (Cytoron Absolute, Ortho Diagnostic Systems, Raritan, NJ, USA). Interferon-gamma (IFN-γ) release was measured in culture supernatants by sandwich ELISA using reagents and protocols obtained from BD Pharmingen, as described previously (8).

Flow cytometry

Analysis of phenotype and activation marker expression was performed as described previously (8,9). Briefly, 1×10^6 cells cultured with or without ConA were harvested and incubated with fluorescein isothiocyanate-, PE-Cy5- or phycoerythrin-conjugated antibodies. Cells were stained for CD4, CD71, CD25 and CD95L (all BD Pharmingen). Staining steps were performed at 4 °C in PBS–EDTA–1% FCS for 30 min. After incubation, cells were washed, fixed in 1% formaldehyde and resuspended; 20 000 events were analysed using Cytoron Absolute

apparatus. Isotype controls (Sigma-Aldrich) that matched fluorochrome were run with each experiment. Additionally, we included MLN and spleen cells from normal rats as controls for intrinsic autofluorescence. On the basis of forward and side light scatter, mononuclear cells were gated in and dead cells were gated out. Isotype-matched control histograms were included to define positivity for each probe as fluorescence that exceeded 98% of controls (16).

Cell cycle analysis

MLNs prepared as described above (1×10^6 cells) were treated with 70% cold ethanol added drop by drop, while vortexing at top speed, and left for 18 h. After incubation, ethanol was removed and cells were resuspended in 0.5 ml of staining buffer (50 μg/ml propidium iodide, 0.1% sodium citrate–50 μg/ml ribonuclease A–300% Nonidet, all reagents from Sigma-Aldrich) for 30 min. Samples were analysed with the Cytoron Absolute technique and cell cycle analysis of DNA profiles was performed using Cylchred software.

Statistical analysis

Data are shown as mean values ± standard deviations. Statistical significance and differences among groups were determined by analysis of variance and Student–Newman–Keuls tests. $P < 0.05$ values were considered statistically significant.

Results

To evaluate the effect of chitosan on proliferation of MLN lymphocytes, cells isolated from normal rats were labelled with CFSE, as this marker attaches to free amine groups of cytoplasmic cell proteins. As the cell divides, retained CFSE label is distributed to each daughter cell. We analysed the effect of chitosan on CD4 T cells by gating of the CD4 subset. Chitosan itself was unable to stimulate proliferation (data not shown). Compared with the 43.4% of proliferating cells in a representative control (Fig. 1a), we found dose-dependent inhibition of CD4+ cell division when lymphocytes were co-cultured with ConA and chitosan (10 or 100 μg/ml). 37.5% and 17.7% of proliferating cells observed represented around 13% and 60%

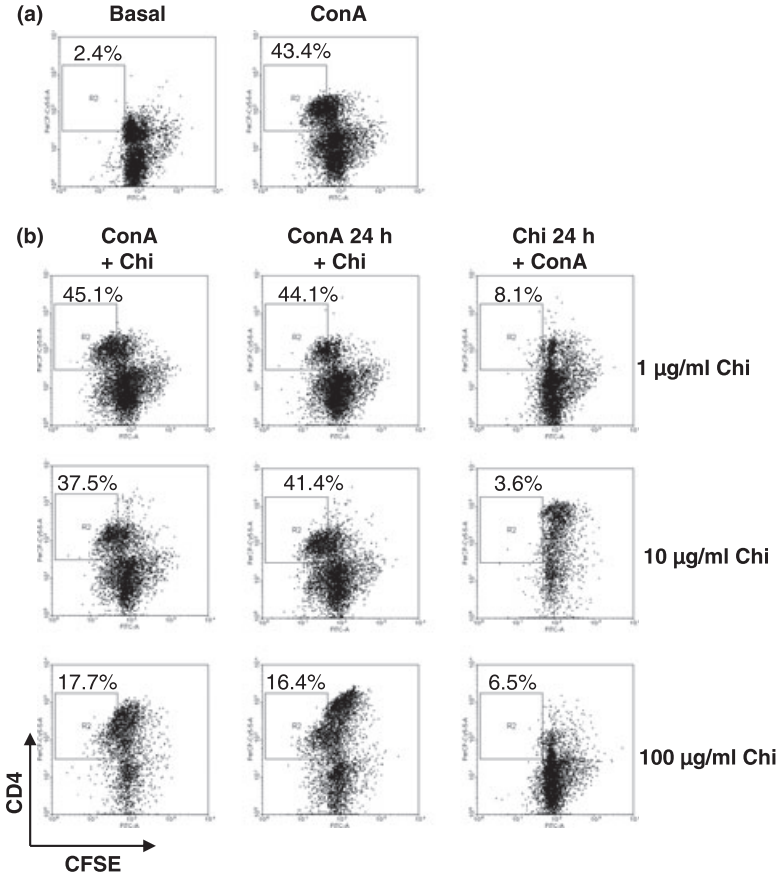

Figure 1. Effect of chitosan on ConA-induced T-cell proliferation *in vitro*. Mesenteric lymph node (MLN) lymphocytes from normal rats labelled with succinimidyl ester of carboxyfluorescein diacetate (CFSE) were stimulated with 2.5 μg/ml of mitogen in presence or absence of chitosan (1–100 μg/ml) at 37 °C in RPMI 1640 medium supplemented with 10% foetal calf serum in a 95% air/5% CO_2 atmosphere for 72 h. After incubation, cells were labelled with anti-CD4 and 20 000 cells were acquired by flow cytometry. Representative dot plots of CFSE-labelled cells representing chitosan-induced inhibition of ConA-induced mitogenesis are shown. Percentage of daughter cells in the box is included. (a) Unstimulated control and ConA stimulated cells; (b) left: ConA + chitosan; middle: ConA for 24 h and then chitosan for further 72 h; right: chitosan for 24 h and then ConA for further 72 h.

inhibition, respectively (Fig. 1b, left). As chitosan could have been interacting with lectin and consequently blocking its activity, cells were first cultured for 24 h with ConA and then treated with the polysaccharide (Fig. 1b, middle). In that condition, we found inhibition only at the highest dose of chitosan tested, with marked reduction of percentage of proliferating cells (16.4%). On the other hand, when cells were treated with chitosan for 24 h and then stimulated with ConA, the percentage of dividing cells dropped dramatically at the three doses evaluated (Fig. 1b, right). In additional experiments, cells were extensively washed after the first 24-h incubation period (either with ConA or chitosan) and before addition of fresh stimuli. No differences were observed when compared with cultures without washing as described above (data not shown).

In the experimental condition described in Fig. 1, we studied expression of activation markers CD25 and CD71 by flow cytometry (Fig. 2a). As can be seen, stimulation with lectin elevated percentage of CD25+ cells (Fig. 2a, c), and the effect was observed even in the presence of chitosan (Fig. 2a, d and e); interestingly, contact with polysaccharide prior to lectin stimulation, that is, Chi24 + ConA (Fig. 2a, f), significantly reduced percentage of CD25+ cells ($P < 0.05$) (Fig. 2a,b). For the CD71 marker, in contrast, increased percentage induced after ConA stimulation showed a slight but consistent modification by polysaccharide treatment, although this was not significant (Fig. 2b).

After prolonged activation, T cells undergo a process termed *activation-induced cell death*, which is mediated by inducible molecules such as Fas ligand (CD95L) (17). Therefore, we evaluated expression of CD95L in CD4+ cells in our cultures. As shown in a representative experiment, the percentage of CD95L lymphocytes varied with different culture conditions (Fig. 2c). Compared to the basal condition, the increment was significant either with ConA or chitosan ($P < 0.05$) (Fig. 2d) and highest values were found in cultures with simultaneous addition of both stimuli (ConA + Chi) or pre-treatment with ConA (ConA24 + Chi) ($P < 0.05$). Interestingly, after pre-treatment with polysaccharide for 24 h (Chi24 + ConA), the percentage of CD95L cells increased, although less compared to cultures with both stimuli or ConA pre-treatment ($P < 0.05$) (Fig. 2d).

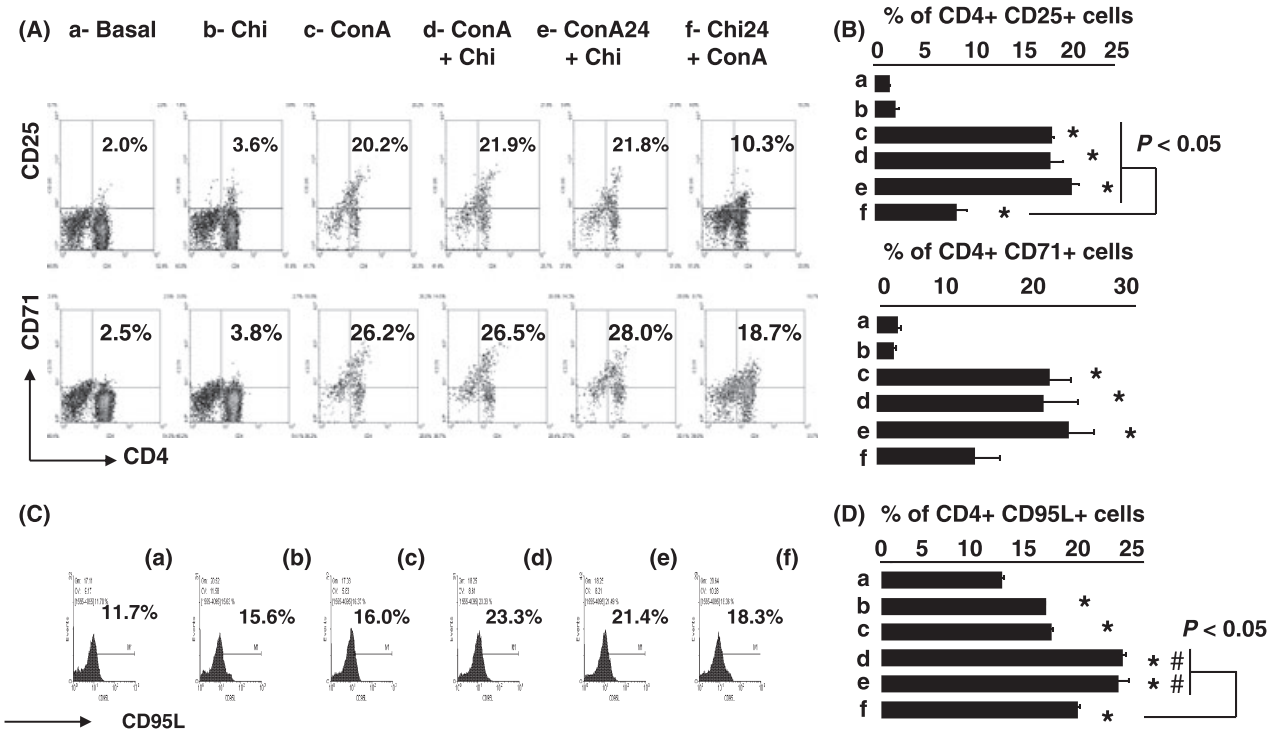

Figure 2. Expression of markers CD25, CD71 and CD95L in lymphocytes stimulated with ConA and chitosan. Mesenteric lymph nodes (MLN) cells (1×10^6 cells/well) cultured for 72 h as described in Fig. 1 were harvested and stained with fluorochrome-conjugated antibodies against CD4, CD71, CD25 and CD95L as described in the Materials and methods section. (A) Representative dot plots of CD25 and CD71 expression in MLN CD4+ cell cultures with (a) medium (basal); (b) chitosan; (c) ConA; (d) ConA 24 together with chitosan; (e) ConA 24 h and then chitosan; (f) chitosan 24 h and then ConA. (B) Mean of CD25 and CD71expression of 6–8 wells per experimental condition from two similar experiments. (C) Representative histograms of CD95L expression in the same experimental conditions of (A). (D) Mean of CD95L expression of 6–8 wells per experimental condition from two similar experiments. *$P < 0.05$ vs. basal; #$P < 0.05$ vs. chitosan or ConA. Other comparisons are indicated with lines in the figure (B and D).

Based on our previous findings in rats fed with chitosan (8,9), we wondered whether this polysaccharide also affects lymphocyte proliferation after oral administration. For this reason, 16 h after feeding a single dose of 1–5 mg chitosan, MLN cells were labelled with CFSE and stimulated with ConA. After 2 days in culture, the percentage of CD4+ cells diluted in CFSE was very low and similar between diluent and groups receiving different doses of chitosan (data not shown). However, after 3 or 5 days we observed a marked reduction in percentage of proliferating CD4+ cells ($P < 0.05$) at the three doses administrated (Fig. 3a) (data not shown). As IL-2 and IFN-γ are Th1-type cytokines produced and released on T-cell activation, we also examined the effect of chitosan on IFN-γ production by MLN cells. ConA strongly stimulated IFN-γ production in cells of diluent group. In contrast, in rats fed with 1–5 mg chitosan, production of pro-inflammatory cytokine IFN-γ was reduced by nearly 13% in culture supernatants ($P < 0.05$).

As the modulatory activity of chitosan at the intestinal mucosa increases after sustained administration of this polysaccharide (8), we also evaluated proliferation of MLN lymphocytes isolated from rats fed 1–5 mg chitosan for 5 consecutive days. Again, previous contact with the polysaccharide at the inductive mucosal lymph nodes reduced proliferation of CD4+ cells (Fig. 3b). However, inhibition observed was similar after feeding single or multiple doses of chitosan. Finally, we assessed *ex vivo* the percentage of MLN lymphocytes in phase G_0–G_1, S and G_2–M of the cell cycle. After a single feeding, percentages were similar in diluent and chitosan groups (Fig. 4). Interestingly, upon continuous administration of polysaccharide, we found an increment in percentage of cells in G_0/G_1 ($P < 0.05$) and a lower percentage of cells in S phase ($P < 0.05$) (Fig. 4). No differences between doses were observed.

Discussion

In the present study, we investigated the effect of 85% de-acetylated chitosan on proliferation of MLN lymphocytes *in vitro* and *in vivo*. Five major findings emerged from this study: (i) chitosan suppresses proliferation of MLN CD4+ cells isolated from normal rats; (ii) interaction with chitosan down-modulates expression of CD25; (iii) chitosan suppresses proliferation of MLN cells after *in vivo* contact; (iv) the polysaccharide reduces secretion of IFN-γ in ConA-stimulated cultures; and (v) chitosan reduces the percentage of CD4+ cells in S phase of the cell cycle after sustained oral administration.

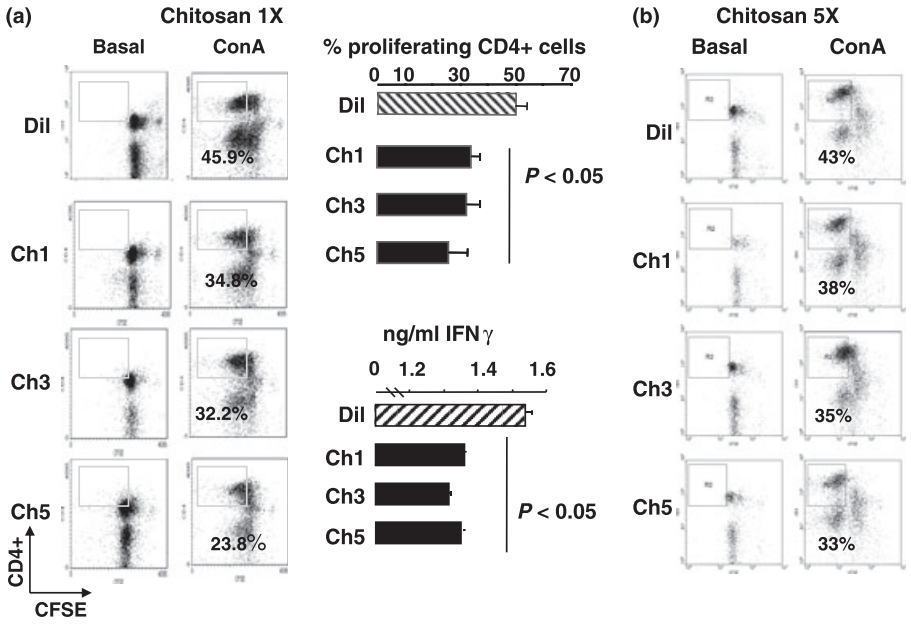

Figure 3. Effect of chitosan on ConA-induced T-cell proliferation *ex vivo*. Mesenteric lymph node (MLN) cells from rats fed a single (a) or five doses (b) of diluent (Dil) or of 1–5 mg chitosan (Ch1–5) were labelled with succinimidyl ester of carboxyfluorescein diacetate (CFSE) and stimulated with 2.5 μg/ml ConA as described in the Materials and methods section. After incubation, cells were labelled with anti-CD4 and 20 000 cells were acquired by flow cytometry. (a) Representative dot plots of CFSE-labelled cells representing chitosan-induced inhibition of ConA-induced mitogenesis are shown. The percentage of daughter cells in the box is included. Bars are means of data from 4–6 rats per treatment. In culture supernatants, the production of INF-γ was evaluated by ELISA. (b) Representative dot plots of CFSE-labelled cells representing inhibition of ConA-induced mitogenesis after sustained administration of chitosan.

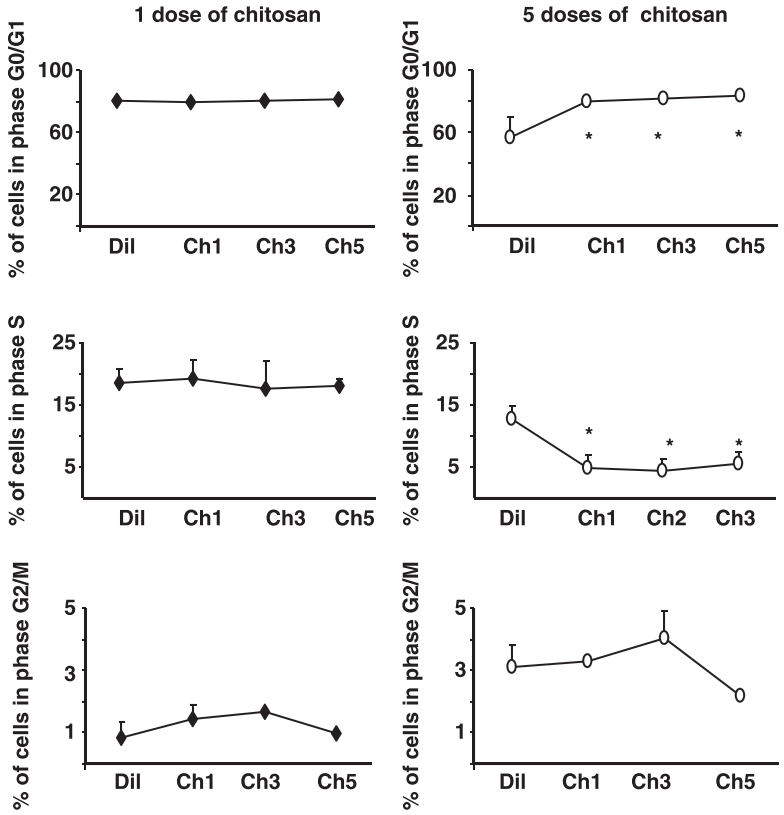

Figure 4. Effect of chitosan on cell cycle regulation of mesenteric lymph node lymphocytes. Mesenteric lymph node cells were isolated from rats fed single or five doses of 1–5 mg chitosan (Ch1–5) or diluent (Dil). DNA contents were analysed by flow cytometry and percentage of cells in G_0/G_1, S, and G_2/M phases of the cell cycle were determined using a established cell DNA analysis software. Data are mean ± standard deviation of two representative experiments with 3–5 rats per condition. $*P < 0.05$ vs. diluent.

Inhibitory activity of several polysaccharides has been demonstrated previously. Water-soluble polysaccharide from *Gynostemma pentaphyllum* herb tea is able to inhibit (by an indirect mechanism), proliferation of human colon carcinoma HT-29 and SW-116 cells (18). Staphylococcal exopolysaccharides inhibit mitogen-stimulated proliferation of human peripheral blood mononuclear cells (19). Furthermore, one polysaccharide from the cryptococcal wall is able to inhibit activation of hybridoma T cells and proliferation of OVA-specific T cells, possibly by a direct effect on T lymphocytes (20). In our experimental conditions, the proliferative response induced with ConA was suppressed by polysaccharide, as indicated by CFSE-labelled cell division data. Lymphocytes become irreversibly stimulated after 18–20 h of exposure to ConA, as the addition of a saccharide, which binds strongly to the lectin 20 h after the addition of ConA, is unable to inhibit proliferation of mouse splenic lymphocytes (21). In agreement, when chitosan was added 16 h after ConA stimulation, we observed mild inhibition only at the highest dose tested. For mouse lymphocytes, all cells do not become committed to stimulation simultaneously, but rather at various times up to 20 h after addition of the mitogen (22). It is possible that MLN CD4+ lymphocytes are heterogeneous in their capacities to respond to ConA, and those cells that become stimulated later are inhibited by higher doses of the polysaccharide. Data obtained from pre-treatment with chitosan confirmed that inhibition of proliferation was not competitive with ConA.

T-cell activation is characterized by phosphorylation of tyrosine kinases at the plasma membrane, calcium mobilization, expression of cell surface activation markers, expression and nuclear localization of transcription factors of activated T cells, production of cytokines (IL-2 and IFN-γ), and, subsequently, cell proliferation (23). Factors downstream of expression of surface markers might interrupt signal transduction pathways before cell entry into S phase. For example, *Agaricus bisporus* lectin triggers early lymphocyte activation cascade(s) although without mitogenic activity (24). Under our experimental conditions, chitosan was unable to affect expression of CD25 and CD71 molecules triggered by ConA. In agreement with recent data (5), only previous contact with the polysaccharide attenuated CD25 expression. CD95L is induced upon TCR/CD3 cross-linking and triggers CD95 signalling and subsequently apoptosis. Increased expression of the death receptor ligand CD95L is suggested to be IL-2 dependent (25). Because IL-2 is one essential factor for proliferation of T cells and sensitization towards activation-induced cell death, a reduced supply of this cytokine could be mediating chitosan activity. In

agreement with this, feeding polysaccharide to the cells induced reduction in levels of IL-2 in spleen and Peyer's patches of rats (8). Recent evidence links reduction in CD95L expression in activated T cells, with the anti-inflammatory effect of glucocorticoids (26). Consistently, our previous observations demonstrate that chitosan contributes to the anti-inflammatory microenvironment in the gut (8,9).

Particular mechanisms maintain immune tolerance to foreign proteins in the intestinal environment, which is exposed to high concentrations of antigens. T cells become activated and proliferate *in vivo* during first contact with tolerizing antigens, but later they become refractory to restimulation *in vitro* (27). Moreover, T cells undergoing tolerance induction do not proliferate at a similar rate or proportion as T cells undergoing sensitization (27,28). The ability of the polysaccharide chitosan to reduce the percentage of proliferating cells and INF-γ secretion on stimulation, supports its tolerizing properties. It has been shown that reduction in proliferation following antigen feeding is due to cell cycle arrest 3 days after antigen feeding (29). Abortive proliferation of T cells could be a default mechanism for initial T-cell responses to antigen (30). It is important to point out here that in terms of proliferation, sustained exposure to the polysaccharide did not enhance its inhibitory effect. Although evidence about polysaccharides is generally lacking, after a single tolerogenic exposure to orally administrated protein antigens, subsequent feeding may not enhance systemic hyporesponsiveness any longer (31).

Tolerized T cells have reduced ability to proliferate and arrest in G_1 phase of the cell cycle. Consistent with inhibited cell division, we found that T-cell proliferation was generally controlled at G_0/G_1 phases, as cell progression through G_1 to S transition was markedly inhibited upon sustained administration of chitosan. Interestingly, in oral tolerance to high doses, antigen-specific T cells stopped dividing yet survived and remained anergic (32). Why some T cells survive deletion to become anergic may reflect differences in characteristics and availability of the antigen or lack of co-stimulatory signals by dendritic cells (33). We have previously shown that chitosan acts by enhancing T helper cell types 2 and 3 microenvironment at mucosal inductive sites (8,9). Early after feeding, an increased number of dendritic cells loaded with the polysaccharide remained, with low expression of co-stimulatory molecules, certainly promoting tolerance induction (9). Moreover, we demonstrated the ability of this polysaccharide to improve tolerance to an articular antigen and to regulate outcome of the inflammatory response in a collagen-induced arthritic model (34). We cannot explain our findings at the molecular level yet. However, activity of this muco-adhesive polysaccharide could involve reduction of protein levels of G_1 phase cell cycle molecules, such as cyclin D3 and cyclin-dependent kinase 6 (CDK6), or an increase in CDK inhibitors, as occurs with other agents that influence T-cell activation and inhibit T-cell cycle progression *in vitro* (35). Recent evidence obtained using synchronized human gastric cancer cells and water-soluble chitosan oligomers has suggested that CDK inhibitors p21 and p27, as well as PCNA (a key molecule involved in DNA replication machinery), are key molecules involved in chitosan-induced suppression of DNA synthesis (7). Studies are under way in our laboratory to disclose the mechanism of chitosan activity *in vivo*.

Here we have described the ability of a polycationic polysaccharide to inhibit mitogen-induced proliferation of mucosal CD4+ cells *in vivo* and *in vitro*, for the first time. Chitosan is a biodegradable agent, widely used as a pharmaceutical excipient and as adjuvant for mucosal vaccination, with an established safety profile in humans. Its ability to limit proliferation of mucosal lymphocytes could be an interesting pathway to contribute to intestinal homeostasis and to promote tolerance to associated antigens.

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