



Basic nutritional investigation

Abrogation of the oral tolerance to ovalbumin in mice by citrus pectin

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Abstract

Objective: We studied the effects of dietary pectins (citrus pectin [CP] and apple pectin) on oral tolerance in mice.

Methods: Pectins (1 mg/d) were administered orally for 2 wk. Tolerance was induced with 20 mg of ovalbumin (OVA). Levels of serum antibodies (immunoglobulin [Ig] G, IgG1, IgG2a, IgE) and delayed type hypersensitivity response determined in footpad tests were measured after subcutaneous injection of OVA with complete Freund's adjuvant. Concentrations of immunoreactive OVA in blood were measured by enzyme-linked immunosorbent assay after feeding the animals 20 mg of OVA. Adhesion and cytokine production (tumor necrosis factor- α , interferon- γ) were measured in peritoneal macrophages.

Results: Oral administration of CP was found to prevent the induction of immune hyporesponsiveness induced by OVA feeding. Animals fed OVA and CP were found to produce similar titers of antigen-specific serum IgG and levels of delayed type hypersensitivity response as those animals not fed OVA. CP increased levels of serum IgG1 and IgE. CP was found to enhance the penetration of immunogenic OVA into the serum. CP (1 mg/d) administered orally for 1 wk was also observed to enhance the adhesion and production of cytokines (tumor necrosis factor- α , interferon- γ) in peritoneal macrophages.

Conclusion: CP administered orally was shown to inhibit oral tolerance. Enhancement of protein antigen penetration to the blood and activation of macrophages were found to precede the inhibitory effect and appeared to mediate it. © 2008 Elsevier Inc. All rights reserved.

Keywords:

Pectins; Citrus pectin; Oral tolerance; Ovalbumin; Anti-ovalbumin antibodies; Peritoneal macrophages; Inflammatory cytokines; Adhesion

Introduction

Oral tolerance (OT) has long been recognized as a physiologic mechanism of immune unresponsiveness to dietary and bacterial antigens that maintain tissue integrity. Breakdown of OT may be the underlying cause of different food-sensitive enteropathies and inflammatory bowel disease [1]. Therefore, the identification of factors that are able to regulate OT is of great interest. It has been hypothesized

that food components may affect the immunogenic potential of ingested proteins [2].

Pectic polysaccharides, which are major constituents in the plant cell wall, are present in the diet and are widely used in the food industry as gelling agents. Moreover, pectins have been shown to possess some immunomodulatory activity [3] and an ability to influence nutrient absorption [4]. Oral administration of the polysaccharide fraction of a dried extract (TJ-48) of the Japanese herb *kampo* to mice resulted in changes in the intestinal immune system [5]. Furthermore, an acidic polysaccharide fraction prepared from a constituent herb of *juzen-taiho-to* has been shown to have protective activity against *Candida* infection in mice [6]. Continuous feeding of the pectin bupleuran, which was prepared from the roots of *Bupleurum falcatum* L., led to enhancement of proliferation of spleen cells [7].

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Citrus pectin (CP), one of the most widely consumed dietary pectins, is known for its ability to decrease plasma cholesterol concentrations [8] and for its influence on metastasis growth [9]. However, the effect of CP on OT has not been studied.

The aim of the present investigation was to study the effect of CP and apple pectin (AP) on OT in mice.

Materials and methods

Materials

Ovalbumin from egg whites (OVA; grade V), AP, thioglycolate, lipopolysaccharide (*Escherichia coli* 0111:B4), *ortho*-phenylene-diamine, phorbol-12-myristate-13-acetate (PMA), and RMPI-1640 medium were purchased from Sigma, St. Louis, MO, USA. CP, complete Freund's adjuvant, bovine serum albumin (BSA), phosphate buffered saline (PBS), and horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin (Ig) G antibodies (Abs) were purchased from MP Biomedicals, Solon, OH, USA.

The galacturonic acid contents of CP and AP were 87% and 75%, respectively, accordingly to certificates of analysis. The degrees of esterification were 56% and 73%, respectively.

Induction of OT and pectin administration

The structure of this study and animal experimental procedures were approved by the ethical committee of the Komi Science Center of the Russian Academy of Sciences on animal care and use.

The mice were fed the cereal-based diet which consisted of 12.7% protein, 5.6% fat and 54.1% carbohydrate, with a total fiber content of 3.7%. The diet was supplemented with a vitamin–mineral premix according to the recommendation of the American Institute of Nutrition (AIN-93M diet) [10].

In experiment 1, female A/HeJ mice (20–25 g) were immunized with 100 μ g of OVA in 50 μ L of complete Freund's adjuvant subcutaneously at the base of the tail. To induce OT, 7 d before subcutaneous immunization, mice were intragastrically given 20 mg of OVA dissolved in 0.2 mL of PBS (tolerant group, $n = 21$) or PBS only (control group, $n = 7$) [11]. Pectins were dissolved in PBS (pH 7.2) up to a concentration of 5 mg/mL. CP and AP solutions obtained were pH 6.7 and 6.6, respectively, and were clear with no visible particulates. Aliquots of the pectic solutions (200 μ L) were administered once daily in the morning to tolerant mice intragastrically through a polyethylene tube for 14 d: 7 d before and 7 d after the OVA gavage ($n = 7$ for CP-treated group, $n = 7$ for AP-treated group). Thus the intake of pectin was 1 mg/d or 40–50 mg/kg. Untreated mice ($n = 7$) received PBS.

To assess OT, delayed type hypersensitivity (DTH) and serum Ab responses were measured in control, tolerant

untreated, tolerant CP-treated, and tolerant AP-treated mice. The mice were injected with 25 μ g of heat-aggregated OVA in 25 μ L of PBS in the right footpad 14 d after subcutaneous immunization; PBS was injected in the left footpad. The volume of the injected paws was measured by using a water-displacement plethysmograph (Ugo Basil, Comerio, Italy) 24 h after injection and was recorded as the difference between the left and right footpads [12].

To determine Ab responses, mice were bled with cardiopuncture after the DTH measurement and blood was centrifuged for 5 min at $400 \times g$. Serum was collected from these samples and stored frozen at -20°C until assayed.

Antibody titers to OVA were assessed using an enzyme-linked immunosorbent assay (ELISA) as described in detail elsewhere [13]. Briefly, flat-bottom polystyrene plates (Costar, NY, USA) were coated overnight at 4°C with 5 μ g of OVA diluted in 0.1 mL of 0.05 mol/L NaHCO_3 buffer (pH 9.6) per well, washed with saline containing 0.05% (v/v) Tween-20, blocked with 1% BSA in PBS, washed again, and then the mouse serum samples with serial dilutions starting at 1:10 were added to the wells. After incubation for 2 h at 25°C , HRP-labeled goat anti-mouse IgG Abs (1/2000) were added to wells and incubated for 2 h at 25°C . The color reaction was developed by the addition of H_2O_2 and *ortho*-phenylene-diamine. The reaction was interrupted after 15 min by the addition of 0.1 mL of 2.5 mol/L HCl and absorbance (OD) was read at 492 nm (Power Wave 200, BioTek Instruments, Winooski, VT, USA). Endpoint Ab titers were expressed as the last dilution producing an OD 0.1 U above negative control values at 492 nm.

Sandwich ELISA was used for measurement of total IgE. Monoclonal affinity-purified anti-mouse IgE antibodies (23G3; eBiosciences, San Diego, CA, USA) were used as the capture Abs for IgE, with biotin-conjugated anti-mouse IgE Abs as the detection Abs. Indirect ELISA was used for measurement of serum OVA-specific IgE. To generate a standard curve, the upper two rows of each plate were coated with the purified goat anti-mouse IgE (23G3; eBioscience). HRP-labeled streptavidin (BD Pharmingen, San Jose, CA, USA) was used for detection. Biotin-conjugated monoclonal Abs specific for $\gamma 1$ (LO-MG1-2) or $\gamma 2a$ (LO-MG2a-9; Acris Antibodies GmbH, Hiddenhausen, Germany) were used for analysis of IgG1 and IgG2a Ab subclasses [14].

Detection of serum-immunoreactive OVA

In experiment 2, mice treated with 1 mg/d of CP or AP for 7 d received 20 mg of OVA and were sacrificed 0.5, 1, 3, 6, or 24 h thereafter. Serum was collected and immunoreactive OVA was measured by indirect competitive ELISA. Microplates with 96 wells (Costar, USA) were coated for 18 h at 4°C with 100 μ L/well of a 25- μ g/mL solution of OVA in carbonate buffer, pH 9.6. The plates were washed four times with PBS containing 0.05% (v/v)

Tween-20 and blocked with 1% BSA in PBS for 60 min. Then, 250- μ L samples of each test serum or standard (0–15 μ g/mL of OVA in saline) were preincubated with 250 μ L of HRP-labeled anti-OVA rabbit polyclonal Abs (Acris Antibodies GmbH) diluted in PBS–Tween-20 for 60 min at room temperature. After preincubation, the samples were added to the wells (100 μ L/well) and incubated for 60 min at 37°C. After incubation and subsequent washing, the color reaction was developed with *ortho*-phenylene-diamine. Absorbances ($\lambda = 492$ nm) of the samples were compared with those obtained from the OVA standards included for each plate [15].

Activity of peritoneal macrophages

In experiment 3, mice treated with 1 mg/d of CP or AP for 7 d were fed 20 mg of OVA and were sacrificed 3 h thereafter. Thioglycolate medium (1.5 mL) was injected intraperitoneally 4 d before OVA feeding. The peritoneal cells were harvested from mice by peritoneal lavage with PBS (5 mL). The cells were washed by centrifugation ($400 \times g$, 10 min) and then suspended in RPMI-1640 medium supplemented with 10% fetal calf serum.

To evaluate the cytokine response, 2×10^6 cells in RPMI-1640 medium supplemented with 10% fetal calf serum per well of flat-bottom microculture plates (Corning) were incubated in a CO₂ incubator at 37°C for 2 h. Monolayer cultures were washed three times with culture medium to remove non-adherent cells. A monolayer of the adherent cells was found to consist mainly of macrophages as estimated microscopically after eosin staining. The culture supernatants were then replaced with fresh RPMI-1640 medium supplemented with 10% fetal calf serum and lipopolysaccharide (1 mg/L) for the stimulation of adherent macrophages and the samples were cultured for 24 h in a CO₂ incubator at 37°C. After incubation, the plates were centrifuged at $800 \times g$ for 10 min and the supernatants were collected and frozen [16].

Levels of tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) were assayed in supernatants by sandwich ELISA according to the manufacturer's protocol (eBioscience and Pierce Biotechnology, Rockford, IL, USA, respectively). Monoclonal affinity-purified anti-mouse TNF- α (clone 1F3F3D4) and anti-mouse IFN- γ (clone XMG1.2) Abs were used as the capture Abs and biotin-conjugated TNF- α (XT3/XT22) and IFN- γ (clone XMG1.2) Abs, respectively, were used as the detection Abs. HRP-labeled streptavidin (BD Pharmingen) was used for detection. Samples were run in duplicate and the results were compared with standard preparations of TNF- α (eBioscience) and IFN- γ (Cytolab/Pepro- tech, Cytolab Ltd., Rehovot, Israel). Levels of sensitivity of the ELISAs were 30 pg/mL for TNF- α and 15 pg/mL for IFN- γ .

To measure cell adhesion, the cell suspensions (0.1 mL) were incubated in a 96-well flat-bottom tissue culture plate in the presence or absence of PMA (0.025 mg/L) as a

stimulating agent at 37°C for 15 min. The wells were washed to remove the non-adherent cells and the adherent cells were fixed in EtOH and stained with azure eosin. The colored material was solubilized in MeOH and the OD of the solution obtained was measured at 650 nm [17].

General methods for chemical characterization of pectin

Galacturonic acid content was determined using an interaction with 3,5-dimethyl phenol in the presence of concentrated sulfuric acid [18]. Descending paper chromatography was run on Filtrak FN-12 and FN-13 papers in the solvent system of *n*-butanol-pyridine-water (6:4:3, v/v/v) followed by detection of sugars with aniline hydrogen phthalate at 105°C. Monosaccharides were identified and quantified as the corresponding alditol acetates [19] by gas–liquid chromatography on a Hewlett-Packard 4890 instrument (USA) equipped with an RTX-1 (0.25 $\times$ 30 m) column in the temperature range of 175–250°C ($\Delta 3^\circ\text{C}/\text{min}$). The sugar quantities were calculated from the peak areas using molar coefficients and *myo*-inositol as the internal standard.

The molecular weights of the pectin macromolecules were analyzed by high-performance liquid chromatography using the following chromatographic system: a pump (SD-200, Dynamax, USA), a column (Shodex Asahipak GS-620 HQ, 7.6 mm $\times$ 30 cm, Shimadzu, Japan) with a pre-column (Shodex GS-26 7B, 7.6 mm $\times$ 5 cm, Shimadzu), a thermostat (CTO-10AS), and refractometer (RID G136A, Shimadzu). Elution was carried out with 0.15 mol/L of NaCl at 40°C with an effluent rate of 0.5 mL/min. Dextran sulfates with molecular masses in the ranges of 36–50 and 400–600 kDa were employed for column calibration.

Complete acidic hydrolysis

Polysaccharides (2–5 mg) were treated with 2 mol/L of trifluoroacetic acid (1 mL) in the presence of *myo*-inositol (0.5 mg/mL) as the internal standard for 5 h at 100°C. The excess of trifluoroacetic acid was removed by repeated evaporation with methanol to complete dryness. The sugars were identified by paper chromatography and gas–liquid chromatography.

Statistical analysis

Results are presented as mean $\pm$ standard deviation. The significance of differences between means was evaluated using Student's *t* test.

Results

Effect of pectins on OT

Feeding of mice with 20 mg of OVA used as a model antigen resulted in subsequent immune hyporesponsiveness

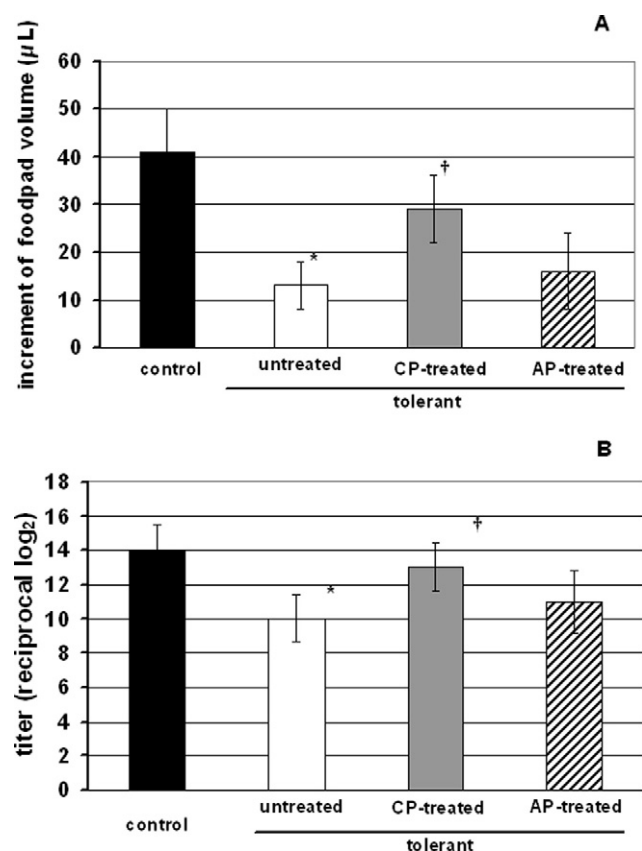


Fig. 1. Effect of CP and AP on oral tolerance induction. (A) Delayed type hypersensitivity reaction and (B) anti-ovalbumin immunoglobulin G production in tolerant untreated (white columns), tolerant CP-treated (gray columns), tolerant AP-treated (striped columns), and control (black columns) mice. Data are presented as mean $\pm$ SD ($n = 7$). * $P < 0.05$ versus controls; [†] $P < 0.05$ versus tolerant untreated. AP, apple pectin; CP, citrus pectin.

to the subcutaneous immunization with the same antigen. Tolerant mice had markedly suppressed DTH responses and reduced OVA-specific serum IgG compared with controls (Fig. 1a,b). CP was found to inhibit OT to OVA. Mice fed OVA and treated with CP for 14 d were found to develop DTH responses and anti-OVA IgG levels that were greater

Table 1

Serum IgE responses induced by subcutaneous immunization with OVA/CFA of mice fed OVA and treated with CP and AP*

Group	Total IgE ($\mu\text{g/mL}$)	OVA-specific IgE ($\mu\text{g/mL}$)
Control	930 $\pm$ 159	6.5 $\pm$ 1.0
Untreated tolerant	590 $\pm$ 48 [†]	4.4 $\pm$ 0.5 [†]
CP-treated tolerant	780 $\pm$ 59 [‡]	6.1 $\pm$ 0.8 [‡]
AP-treated tolerant	630 $\pm$ 150	4.8 $\pm$ 1.5

AP, apple pectin; CFA, complete Freund's adjuvant; CP, citrus pectin; IgE, immunoglobulin E; OVA, ovalbumin

* Data are presented as mean $\pm$ SD ($n = 7$).

[†] $P < 0.05$ versus control.

[‡] $P < 0.05$ versus untreated tolerant.

Table 2

Serum anti-OVA IgG subclass responses induced by subcutaneous immunization with OVA/CFA of mice fed OVA and treated with CP and AP*

Group	IgG1 (reciprocal log ₂)	IgG2a (reciprocal log ₂)
Control	13.1 $\pm$ 1.3	8.5 $\pm$ 2.0
Untreated tolerant	5.7 $\pm$ 2.0 [†]	4.8 $\pm$ 2.1 [†]
CP-treated tolerant	13.4 $\pm$ 1.7 [‡]	7.1 $\pm$ 2.8
AP-treated tolerant	3.6 $\pm$ 2.4	3.0 $\pm$ 2.4

AP, apple pectin; CFA, complete Freund's adjuvant; CP, citrus pectin; IgG, immunoglobulin G; OVA, ovalbumin

* Data are presented as mean $\pm$ SD ($n = 7$).

[†] $P < 0.05$ versus control.

[‡] $P < 0.05$ versus untreated tolerant.

than those of untreated tolerant animals. A similar pattern was seen when serum anti-OVA IgE levels were examined (Table 1). AP failed to influence OT.

Treatment with CP was conducted to change the pattern of IgG subclasses. The induction of OT was found to decrease levels of IgG1 and IgG2a Abs (Table 2). CP was demonstrated to increase levels of IgG1 two-fold with little effect on IgG2a response. This pattern of response was found in every mouse tested.

Effect of pectins on concentration of OVA in blood

The first property of CP that we deemed likely to be important in the inhibition of OT was the effect on the uptake of OVA from the intestine. To test this, the level of immunogenic OVA was measured in the serum after feeding the animals 20 mg of OVA. As shown in Figure 2, mice fed OVA alone had detectable amounts of OVA 0.5, 1, 3, and 6 h after protein ingestion. Serum OVA levels were three- and four-fold higher 3 and 6 h, respectively, after OVA feeding in mice treated with CP. Thus, CP appeared to

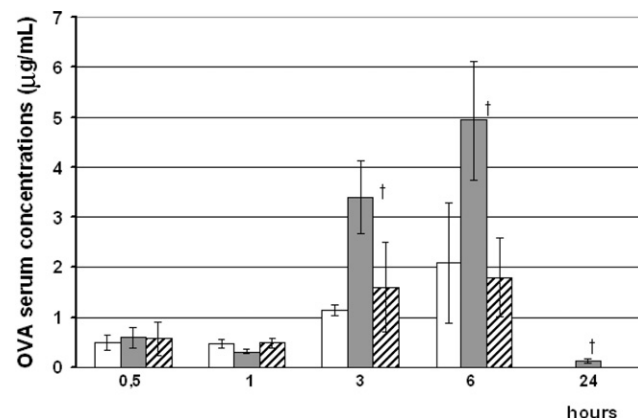


Fig. 2. Effect of citrus and apple pectins on the concentration of immunogenic OVA in serum after OVA gavage. Data for untreated (white columns), citrus pectin-treated (gray columns), and apple pectin-treated (striped columns) mice are presented as mean $\pm$ SD ($n = 7$). [†] $P < 0.05$ versus untreated group. OVA, ovalbumin.

Table 3

Effect of CP or AP on activity of peritoneal macrophages of mice that received 20 mg of OVA orally*

Group	Cell amount (10^6)	Cell adhesion ($OD_{650} \times 1000$)	
		Spontaneous	PMA-stimulated
Untreated	10.4 ± 2.7	141 ± 18	245 ± 35
CP-treated	$15.9 \pm 5.9^\dagger$	$174 \pm 5.5^\dagger$	$301 \pm 23^\dagger$
AP-treated	10.9 ± 2.3	144 ± 9	250 ± 7.3

AP, apple pectin; CP, citrus pectin; OD_{650} , absorbance measured at 650 nm; OVA, ovalbumin; PMA, phorbol-12-myristate-13-acetate

* Data are presented as mean $\pm$ SD ($n = 7$).

$^\dagger P < 0.05$ versus untreated group.

increase the ability of the intestine to take up associated protein. AP had no effect on the OVA concentration in the blood.

Activity of peritoneal macrophages after pectin exposure

To determine the effect of CP or AP on the activity of macrophages, we examined the adhesion and cytokine production of the peritoneal macrophages of mice treated with pectin for 7 d and fed 20 mg of OVA. OVA feeding had no effect on peritoneal leukocyte count, cell adhesion, and cytokine production; all of these parameters were the same in tolerant and control mice 3 h after OVA administration (data not shown). The total number of peritoneal leukocytes was significantly increased in the CP-treated group compared with untreated mice (Table 3). CP was determined to enhance spontaneous and PMA-stimulated cell adhesion (Table 3). The levels of TNF- α and IFN- γ produced by peritoneal macrophages were elevated in mice treated with CP when compared with untreated mice. AP did not influence cytokine secretion or adhesion of macrophages (Table 4).

General characterization of pectins

The observed monosaccharide composition of the pectins studied was comparable to the datasheets provided by manufacturers. CP was shown to consist mainly of galacturonic acid residues (58%) and residues of glucose (37%) with minor residues of rhamnose (0.5%), arabinose (0.5%), and galactose (2.1%). AP was determined to consist mainly of galacturonic acid residues (77%) with residues of glucose (2.9%), rhamnose (1.5%), arabinose (4.2%), xylose (1.9%), mannose (0.2%), and galactose (8.3%). Both pectins were found to contain polysaccharide chains with molecular weights 100–300 kDa (80%) and >400 kDa (20%).

Discussion

The principal aim of this study was to investigate the effect of dietary pectins on OT induction in mice. The dose of pectin used was chosen to approximate the estimated

pectin intake in humans. In epidemiologic studies, median intake of pectin was 1–7 g/d or 10–90 mg/kg [20,21]. Moreover, pectins have been found to exhibit antiallergic activity at doses of 5–12 mg/kg [22,23] and to possess an immunostimulatory effect at the dose of 50 mg/kg [7,24]. The activation of immune system is well known to inhibit OT [25]. Therefore, it was assumed that pectins affect OT when given at the dose of 50 mg/kg. The data demonstrate that CP (50 mg/kg) administered orally with OVA inhibits the development of OT to that protein. This dose is much higher than the effective doses of other known compounds influencing OT. Cholera enterotoxin and heat-labeled toxin from *E. coli* have been shown to abrogate OT with a single, orally administered dose of 10 μ g [26,27].

The DTH response and levels of serum antigen-specific IgG are commonly used for OT assessment [28]. CP was found to increase DTH and anti-OVA IgG, indicating an enhanced immune response to fed OVA in mice that received CP. Enhanced DTH reactions in mice in response to OVA as a thymus-dependent antigen revealed the stimulatory effect of CP on T-helper type 1 (Th1) lymphocytes. This is relevant because, in enhanced DTH reactions, accessory cell types are required for expression. DTH requires the specific recognition of a given antigen by memory Th1 cells, which subsequently proliferate and release cytokines. In turn cytokines increase vascular permeability and induce vasodilatation and accumulation of inflammatory cells (neutrophils, macrophages) [29]. At the same time, the recovery of anti-OVA IgG, IgG1, and IgE serum levels induced by CP in tolerant mice indicates a humoral response to stimulation and suggests the activation of Th2 lymphocytes. Dual Th1- and Th2-potentiating activity has been described previously for polyacryl starch microparticles [30].

Citrus pectin appeared to increase the ability of the intestine to take up associated materials as confirmed by the higher levels of circulating OVA after feeding in mice treated with CP. Consistent with previous reports, we detected some amount of OVA even at 0.5 h after feeding [31]. CP was found to enhance the concentration of immunogenic OVA in the serum and to prolong its residence time in the circulation. We hypothesize that the CP-mediated increase in the OVA level might be very important for OT abrogation. These findings are consistent with those from previous reports that described increased antigen penetra-

Table 4

Effect of CP or AP on cytokine production by peritoneal macrophages of mice that received 20 mg of ovalbumin orally*

Group	TNF- α (pg/mL)	IFN- γ (pg/mL)
Untreated	59 ± 3	138 ± 10
CP-treated	$84 \pm 8^\dagger$	$184 \pm 15^\dagger$
AP-treated	61 ± 2	142 ± 8

AP, apple pectin; CP, citrus pectin; IFN- γ , interferon- γ ; TNF- α , tumor necrosis factor- α

* Data are presented as mean $\pm$ SD ($n = 7$).

$^\dagger P < 0.05$ versus untreated group.

tion through the intestinal tract in allergic patients and that the presence of that immunogenic antigen that had penetrated through the intestinal barrier could increase severe allergic reactions [32,33]. Abrogation of OT to food protein can enhance the risk of food allergies. Dietary pectins such as CP manufactured with added glucose are vastly used in the food industry and presented in diets as nutrients. Therefore, they should be safely ingested.

We have also shown that oral administration of CP activated innate immunity as assessed by enhancement of TNF- α and IFN- γ production and increased adhesion of peritoneal macrophages. These cytokines are known as key mediators in the response to infection and inflammatory stimuli and indicate the activation of innate immunity. The data obtained are in agreement with previous studies, which determined that the activation of the immune system during tolerogen ingestion prevents OT development. DePaolo et al. [34] reported that reduced expression of chemokine (C-C motif) ligand 2 on macrophages shifted the gut-associated lymphoreticular tissue cytokine balance toward interleukin-12 production and the promotion of the immunity, which resulted in abrogation of high-dose OT induction. Blocking of monocyte chemotactic protein-1, which inhibits production of mucosal interleukin-12 and T-cell activation, also prevented OT induction [35]. Moreover, stimulation of reticuloendothelial system activity prevented the induction of tolerance to OVA [25].

Because AP failed to influence OT, structural features of CP are assumed to mediate its immunostimulatory effect. However, we did not identify these structural features in the present work. The dramatically higher glucose in CP might be explained by the technology of dietary pectin manufacturing. Glucose is usually added to dietary pectins to enhance their gelling and viscosity properties. We speculate that the presence of glucose may determine the conformation of CP, which in turn may determine its effect on OT.

Conclusions

The present study has demonstrated that CP administered orally inhibited OT in mice. Enhanced protein antigen penetration into the blood and activation of macrophages were found to precede the inhibitory effect and appeared to mediate it.

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