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Oral administration with chitosan hydrolytic products modulates mitogen-induced and antigen-specific immune responses in BALB/c mice

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Abbreviations:

GM-CSF, granulocyte macrophage colony stimulating factor; IFN-γ, interferon-γ; IL-1, interleukin-1; IL-4, interleukin-4; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; NO, nitric oxide; LMWC, low-molecular-weight chitosan; LPS, lipopolysaccharides; ConA, concanavalin; OVA, ovalbumin

Abstract

The aim of this study was to investigate whether oral administration in BALB/c mice with chitosan hydrolytic products including chitosan hydrolysate, LMWC and a chitooligosaccharide mixture (oligomixture), modulates mitogen-induced and antigen-specific immune responses. A water-soluble chitosan hydrolysate was obtained via cellulase degradation of chitosan, and a LMWC and the oligomixture were separated from this hydrolysate. In non-immunized mice, both the chitosan hydrolysate and oligomixture significantly increased the phagocytic activity of peritoneal macrophages. Three chitosan hydrolytic products significantly increased the mitogen-induced proliferation of splenocytes and Peyer's patch (PP) lymphocytes. LMWC and oligomixture up-regulated IFN-γ secretion, and induced predominantly Th1 cytokine secretion in splenocytes. In antigen-specific immunity, similar effects of the chitosan hydrolytic products were observed on augmenting ovalbumin (OVA)-, as well as mitogen-, induced proliferation of splenocytes harvested from OVA-immunized mice. Interestingly, oligomixture was the most potent chitosan hydrolytic product to elicit OVA-specific IgM, IgG, and IgA production, while LMWC was the most potent one to elevate splenic IFN-γ production and IFN-γ/IL-4 (Th1/Th2) ratio. These results provide the distinct immunomodulatory properties of chitosan hydrolytic products in response to mitogens and specific antigen, paving the way for further development and application of dietary chitosan hydrolytic products against immune disorders and infection.

Keywords: antibody; chitosan hydrolysate; immunomodulation; lymphocyte; phagocytic activity

1. Introduction

Innate immunity is a nonspecific defense mechanism that acts as the host's first line of defense against infection [1] and is rapidly initiated upon pathogen infection. Activated phagocytes, such as macrophages, neutrophils, and dendritic cells, recognize and eliminate pathogens, either by attacking or engulfing larger pathogens [2]. These cells are also important mediators in the activation of the adaptive immune system [3], which will respond more vigorously following repeated exposure to the same pathogen/antigen. The main weapons of this immune system are antibodies, secreted by B lymphocytes and circulating in the blood (humoral immunity), and T lymphocytes, able to either destroy directly infected cells or activate macrophages via the secretion of IFN-γ to kill pathogens (cell-mediated immunity) [4].

Gut-associated lymphoid tissue (GALT) is one of the largest lymphoid organs in the body, containing up to 70% of the body's immunocytes, and plays an important role in the immune system [5, 6]. Typical GALT structures can be seen in Peyer's patches (PPs), the aggregated lymphoid follicles in the small intestinal mucosa. PPs are covered in M (microfold) cells, which lack microvilli and facilitate antigen translocation and the presentation of luminal antigens to sub-epithelial professional immune cells [7]. After antigen presentation, lymphocytes in PPs differentiate into effector lymphocytes and B cells to produce immunoglobulin A (IgA) with the activity to neutralize pathogen/antigen [8].

Chitosan, a partially deacetylated chitin [poly-β-(1→4) N-acetyl-D-glucosamine] has immune-enhancing activity, as it stimulates macrophages to produce IL-1, IL-6, TNF-α, NO, and GM-CSF [9, 10]. These functions are initiated when chitosan binds to the specific receptors on macrophages [11-13]. Both the chemical structure and the molecular size of chitosan may affect this binding efficacy and modulate the host immune responses [14, 15].

Although chitosan has strong biological functions, its water-insolubility makes it disadvantageous as an immunotherapeutic agent. Water-soluble chitooligomers — hexamers of N-acetyl chitohexaose and chitohexaose in particular — have attracted much attention for their immune-enhancing effects [16, 17]. However, the very high cost of these hexamers limits their application. In our previous study, a water-soluble chitosan hydrolysate with immuno-activity was obtained by cellulase degradation of chitosan [18]. A LMWC (20 kDa) and a chitooligosaccharide mixture [designated as oligomixture, containing sugars with a degree of polymerization (DP) of 1–6] isolated from this chitosan hydrolysate were shown to have different stimulatory effects on the cell proliferation and IgM secretion of human hybridoma HB4C5 cells [18]. Also, in the RAW 264.7 murine macrophage cell line, application of this oligomixture enhanced NO production, markedly suppressed superoxide production, stimulated phagocytic activity, and promoted the expression of dendritic cell surface markers, while application of LMWC produced the opposite effect [13].

In the present study, chitosan hydrolytic products including the chitosan hydrolysate and its two components, LMWC and oligomixture, were orally administered to BALB/c mice, and their immunomodulatory activities on mitogen-induced and antigen-specific immune responses were evaluated. Changes in splenocyte and PPs lymphocyte cell proliferation, phagocytic activity of peritoneal macrophages, serum antibodies, and splenocyte cytokine secretion were monitored in mice, which had received orally administered chitosan hydrolytic products with or without OVA immunization.

2. Materials and Methods

2.1. Reagents and animals

LPS, ConA, OVA, 3-(4,5-dimethylthiazol-2y1)-2,5-diphenyltetrazolium bromide

(MTT), peroxidase-conjugated goat anti-mouse IgG, and peroxidase-conjugated goat anti-mouse IgM were purchased from Sigma Chemical Co. (Gillingham, UK). Goat anti-mouse IgA, peroxidase-conjugated goat anti-mouse IgA, mouse IgM, and mouse IgG were purchased from Rockland (Gilbertsville, PA, USA). Goat anti-mouse IgM, and goat anti-mouse IgG (H + L) were purchased from Pierce (Rockford, IL, USA). Penicillin-streptomycin solution (100X) and bovine serum albumin (BSA) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Fetal calf serum (FCS) was purchased from PAA Lab. (Linz, Austria). Enriched RPMI 1640-Dulbecoo's-Ham's F12 (eRDF) was purchased from Kyokuto Pharmaceutical Kogyo Company (Tokyo, Japan). *Saccharomyces cerevisiae* BCRC 21607 was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). BALB/c mice were obtained from the Animal Center at the College of Medicine, National Taiwan University (Taipei, Taiwan).

2.2. Preparation of chitosan:

Chitin was isolated from shrimp (*Solenocera prominentis*) shells using the methods reported by Tsai and Su [19]. The isolated chitin powder was further deacetylated with 50% NaOH in a 140°C oil bath for 1 h to obtain chitosan with a molecular weight of 300 kDa and a 95% degree of deacetylation (DD 95), which were evaluated using size-exclusion high-performance liquid chromatography (SE-HPLC) [20] and a colloid titration method [21], respectively.

2.3. Production of chitosan hydrolysate and component separation.

According to the protocol of Wu and Tsai [18], 100 g DD95 were added to a 5 L fermenter (CMCF-5, Chin Chi Co., Taipei, ROC) containing 2000 mL acetic acid-bicarbonate buffer (pH 5.2), followed by the addition of cellulase (10 U/mL). After digestion at 55°C and rotation at 125 rpm for 9 h, the hydrolysate was neutralized and centrifuged (12,000 × g, 30 min). The supernatant (designated as chitosan hydrolysate in this study) was added to an equal volume of methanol and separated into two fractions: the chitooligosaccharide mixture (designated as oligomixture) in the upper fraction and LMWC in the precipitate [18]. The chitosan hydrolysate, LMWC, and

oligomixture were lyophilized and stored at 25°C prior to use. The molecular weight and deacetylation degree of LMWC was 20 kDa and 83%, respectively, which were analyzed by using size-exclusion high-performance liquid chromatography (SE-HPLC) [20] and a colloid titration method [21], respectively. The oligomixture was composed of chitooligosaccharides with DP of 1-6, and the weight percentages for these sugars were 36.8%, 9.5%, 2.7%, 2.1%, 16.3% and 32.6%, respectively [18]. All samples were confirmed to be endotoxin-free using a Pyrotell® Limulus Amebocyte Lysate kit (Associates of Cape Cod Inc., E. Falmouth, MA, USA) [13].

2.4. Animals and experimental design

Conventional female BALB/c mice (4 weeks old) were obtained from the Animal Center at the College of Medicine, National Taiwan University (Taipei, Taiwan). The mice were housed six to a cage and fed ad libitum. The cages were kept in a humidity, light and temperature-controlled environment. Approval for the present study was obtained from the National Taiwan Ocean University Institutional Animal Care, and the mice were handled and euthanized according to its guidelines.

The mice were fed a normal diet for 1 week, and were then divided into four groups, each consisting of 10 mice, and each control or experimental compound was administered orally as follows: 0.5 mL saline (for control group), chitosan hydrolysate, LMWC, or oligomixture at a dosage of 500 mg/kg body weight (for experimental group) 6 times per week for 4 weeks via a gastric tube. Sera were collected in the 2nd and 4th weeks for antibody measurement. Animals were euthanized on dry ice and peritoneal macrophages were collected. The spleen and PP, as a systemic and intestinal immune barometer, respectively, and were removed and processed, as reported in section 2.5.2.

Another animal feeding experiment was conducted to determine specific immune-modulation, in which mice from both the control group and experimental groups were immunized via intraperitoneal injection of 0.2 mL OVA solution (50 μg/mL) in the 2nd week. A boosting injection was given in the 4th week. Sera were collected in the 5th week for OVA-specific antibody measurement. The mice were euthanized 1 week after the boosting injection, and the splenocytes and PP cells were collected for proliferation assay.

2.5. Preparation of peritoneal macrophages.

The murine peritoneal macrophages were collected based on the method described

by Lewis, et al. [22]. Briefly, a 5 cm incision was made in the abdominal wall of the euthanized mice, followed by injection of 3 mL Hanks balanced salt solution (HBSS) containing 1X penicillin-streptomycin solution. Peritoneal exudates were harvested from peritoneum lavage. The cells were washed twice with HBSS, re-suspended in eRDF supplemented with 10% FCS, and incubated at 37°C for 1 h. The adherent cells were used in the phagocytic activity assay.

2.5.1. Phagocytic activity of macrophages.

The phagocytic activity of peritoneal macrophages was evaluated using the suspension assay as described by Bin-Hafeez, et al. [23], with minor modifications. Briefly, a 0.5 mL aliquot of cells (2×10^6 cells/mL) was mixed with 0.5 mL of 10% FCS opsonized yeast (*Saccharomyces cerevisiae*) cells (2×10^7 cells/mL). The mixture was incubated at 37°C for 1 h. After incubation, 50 μL of this mixture were smeared on a glass slide, air dried, and stained with Giemsa stain. The macrophages that phagocytized yeast cells were counted using a light microscope. Two hundred cells from each animal were examined for each assay.

2.5.2. Lymphocyte proliferation assay.

The murine spleen and PP cells were suspended in 5 mL eRDF with 10% FCS added. After the addition of an equal volume of Ficoll-paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and centrifugation (400 x*g*, 20 min), the white opaque mononuclear cell fraction from the interface between the medium and the Ficoll-paque was collected, and washed 3 times with 5 mL eRDF medium (containing 10% FCS). One hundred microliters of the splenocyte and PP lymphocyte cell suspensions (2×10^6 cells/mL) were added to the wells of microtiter plates, followed by the addition of 20 μL of Con A (15 μg/mL), LPS (30 μg/mL), or OVA (100 μg/mL). After incubation at 37°C for 48 h, the viable cells were measured via MTT assay [21]. The proliferation index was calculated as follows.

$$\text{Proliferation index} = (A_{\text{sample}} / A_{\text{control}}) \times 100\ \%$$

A_{sample}: absorbance of cell from mice receiving sample in MTT assay.

A_{control}: absorbance of cell from control mice in MTT assay.

2.5.3. Determination of cytokines by ELISA.

The splenocytes were cultured in eRDF medium containing 10% FCS with/without

addition of LPS (5 μg/mL), Con A (2.5 μg/mL), or OVA (100 μg/mL) at 37°C for 48 h. The amounts of IFN-γ and IL-4 in the supernatant of splenocyte cultures were quantified using their respective DuoSet ELISA Development kits (R&D Systems, Minneapolis, MN, USA) as directed by the manufacturer.

2.5.4. Determination of antibodies by ELISA.

The total antibodies or OVA-specific antibodies in sera were quantified according to the method of Wu and Tsai [18], with minor modification. Two hundred microliters of 10^3-fold-diluted goat-anti-mouse antibodies or 20 μg/mL OVA in 50 mM carbonate coating buffer (pH 9.6) were added to the wells of the microtiter plate and incubated overnight at 4°C. After washing with phosphate buffered saline (PBS; 0.2 g KH2PO4, 1.15 g Na2HPO4, 0.2 g KCl, and 8 g NaCl in 1 L of deionized water) containing 0.05% Tween-20 (PBST), 100 μL of the sample serum were added to the well. The plates were incubated at 37°C for 1 h, washed, and 200 μL of peroxidase-labeled goat anti-mouse antibodies were added to each well (1:3000 dilution with PBST plus 1% BSA). After incubation at 37°C for 1 h, the plates were washed again, 100 μL of ABTS (from an assay kit obtained from KPL, Gaithersburg, Germany; prepared by mixing equal volumes of solutions A and B, as described in the manufacturer's instructions) were added and the plates were incubated at 37°C for 15 min. Finally, 100 μL of 1.5% oxalic acid was added to each well and the absorbance was measured at 410 nm.

2.6. Statistical analysis.

The results are presented as the mean ± SD. Data were analyzed using the statistical analysis system program. Statistical comparisons were made using ANOVA and Duncan's tests. Differences of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

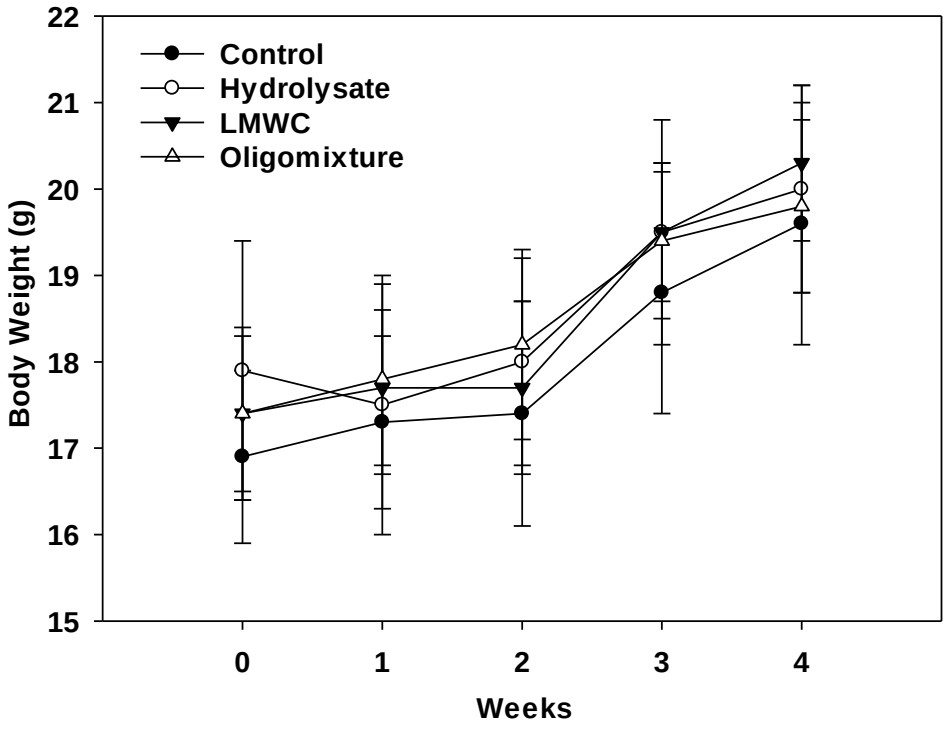
3.1. In vivo immunomodulatory effects of chitosan hydrolytic products on phagocytic

activity of macrophages and lymphocyte responses

During the 4 weeks of the oral administration experiment, the body weights of all mice slightly increased, and the gains in body weight were not significantly different between the experimental groups and the control (Fig. 1A). After the oral administration of chitosan hydrolytic products for 4 weeks, the percentage of peritoneal macrophages with phagocytic activity were 20.6 ± 5.9, 8.1 ± 4.0, and 14.5 ± 4.1% from hydrolysate, LMWC, and oligomixture, respectively, while that of the control group was 6.1 ± 1.6%. Chitosan hydrolysate and oligomixture increased the phagocytic activity of macrophages (Fig. 1B).

Macrophages, which act as phagocytic, microbicidal, and tumoricidal cells, play a critical role in the host's first line of defense against infections. In a previous study, chitosan hydrolysate and its oligomixture component were demonstrated to enhance NO production in IFN-γ-treated murine RAW264.7 macrophages in vitro [13]. In the present study, the chitosan hydrolysate and oligomixture were further demonstrated to enhance the phagocytic activity of peritoneal macrophages in mice.

(A)



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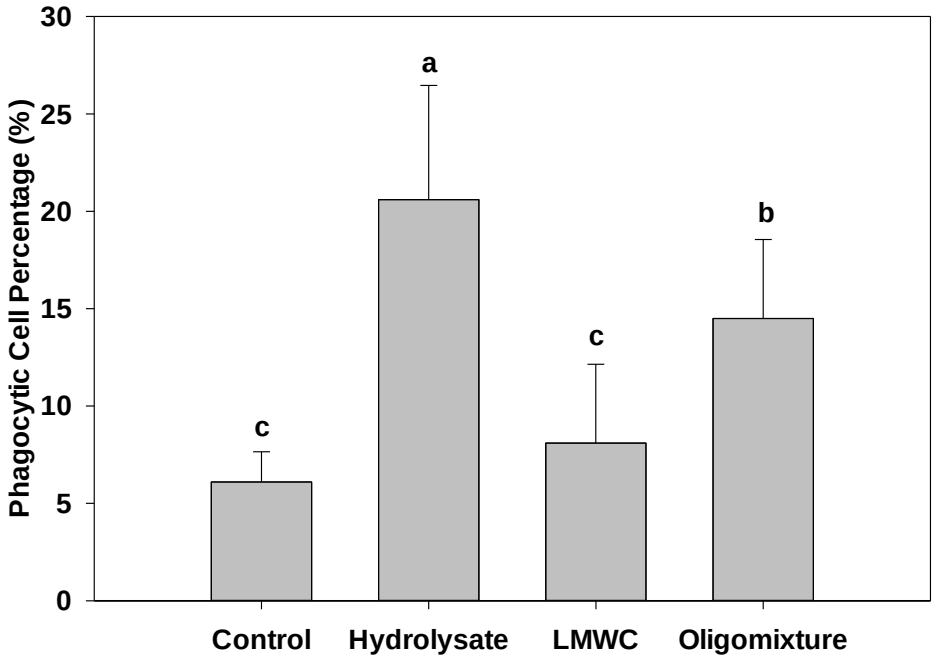
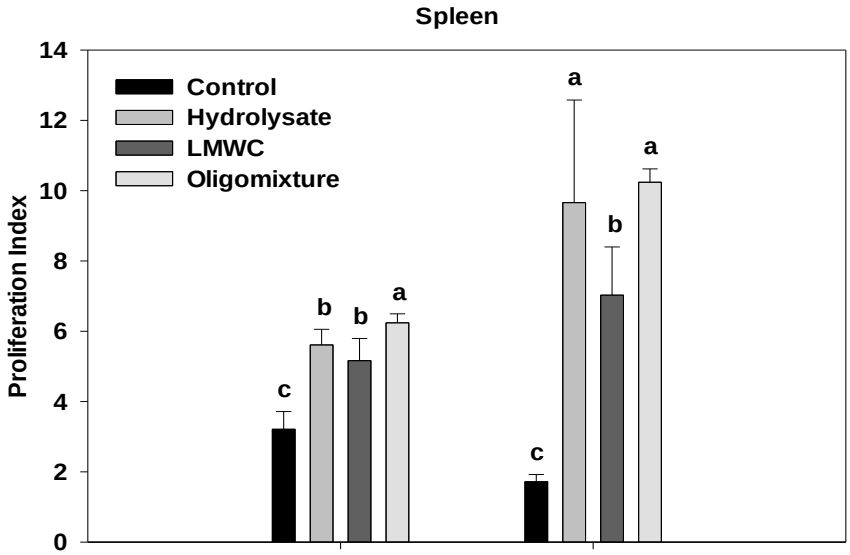


Fig. 1. Chang et al.

In addition to phagocytic activity of macrophages, lymphocyte proliferative responses also correlate with the resistance against infection. Therefore, we investigated the lymphoproliferative responses of splenocytes and PP lymphocytes harvested from chitosan hydrolytic products-treated mice in response to the mitogens for T-cell (ConA) and B-cell (LPS). The chitosan hydrolysate, LMWC, and oligomixture significantly increased the proliferation index of splenocytes stimulated by the mitogens (Fig. 2A), and significantly increased the proliferation index of PP lymphocytes stimulated by Con A (Fig. 2B). James and Zeitz [24] indicated that lymphocytes in the intestinal mucosa first interact with antigens in the organized lymphoid tissues (PPs and lymphoid follicles in the colon), and after maturation they migrate through the mesenteric lymph nodes to reach systemic circulation. Porporatto, et al. [25] demonstrated the presence of chitosan in PPs after oral administration, and they further showed that chitosan in PPs can up-regulate the major histocompatibility complex (MHC) class II molecules of antigen-presenting cells (APCs). In consistent with the above studies, our results further indicate that chitosan hydrolytic products are potential to up-regulate T-cell immune responses in PP and T-cell and B-cell immunity in the spleen, indicating that the immune cells regulated by hydrolytic products at the intestine potentially migrate by circulation to the spleen to further modulate systemic immunity. Maeda and Kimura

[26] reported that S180-bearing mice treated with LMWCs (21 and 46 kDa) and chitooligosaccharides showed increased antitumor activity and induced activation of macrophages through the production of cytokines such as IFN-g, IL-12, and IL-18 from intestinal intraepithelial lymphocytes and splenic lymphocytes. However, larger MW chitosans (130 or 650 kDa) had no such effect. This suggests a possible mechanism by which the chitosan hydrolytic products administered to the gastrointestinal tract were able to elicit widespread systemic responses, as indicated by the increasing proliferative index of splenocytes and PP cells in the present study.

(A)



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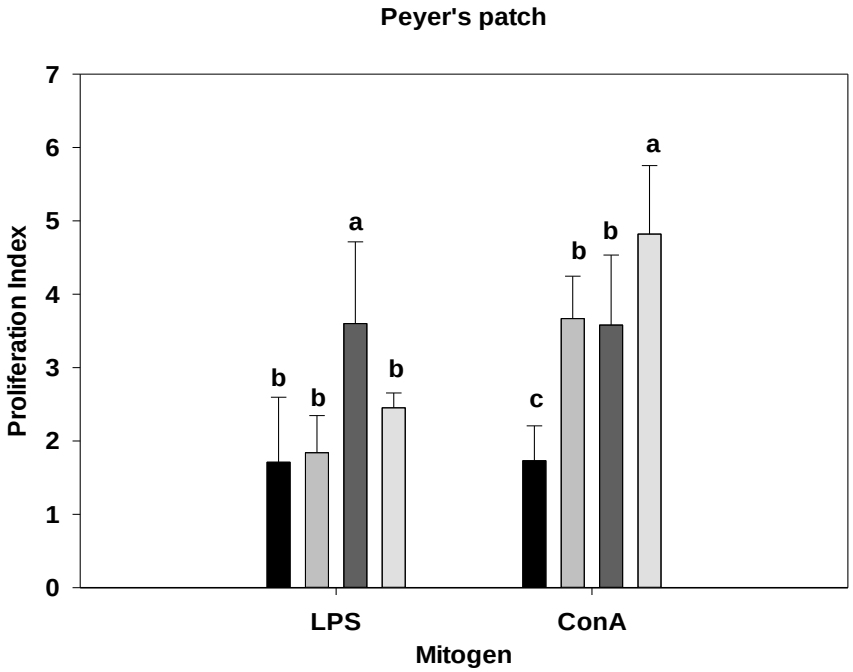


Fig. 2. Chang et al.

It has been reported that chitin could be served as a mitogen to induce the differentiation of B lymphocytes into antibody-forming cells *in vitro* [27]. Recently, chitin, chitosan and their derivatives have been utilized as adjuvants to enhance antibodies production [28-30]. As antibody production represents an end-point affecting response of T lymphocyte-mediated antigen recognition, we further investigated whether oral administration with chitosan hydrolytic products influence the levels of antibody production. We used ELISA to measure the total levels of IgG, IgA, IgM, and IgE in sera in the 2nd and 4th weeks of administration, as shown in Fig. 3. The levels of IgA, IgG, and IgM were significantly increased by administration of oligomixture. LMWC increased the IgG and IgM levels, while the hydrolysate increased the IgM level compared with the control. Based on the results of B-cell proliferation and antibody production, chitosan hydrolytic products, especially oligomixture, are promising to up-regulate humoral immunity. Noticeably, the level of IgE production was not influenced by chitosan hydrolytic products (Fig. 3), indicating a limited risk of chitosan hydrolytic products to induce type 1 hypersensitivity [31].

In general, naive T cells recognize antigen in the context of MHC molecules via TCR complex and develop into armed affecting T cells which can largely be divided into T helper (Th) cells. Among the subsets of Th cells, Th1 cells secrete IFN-γ and IL-2 to augment cell-mediated immune responses. In contrast, Th2 cells uniquely secrete IL-4, IL-5, and IL-6 to help the antibodies production by B cells, and mediate the

responses against extracellular parasites [32].

To investigate the effect of chitosan hydrolytic products on the production of IFN-γ and IL-4, the splenocytes were cultured with or without mitogen for 48 h, and the level of cytokine secretion in supernatants were measured by ELISA. The levels of spontaneous secretion of IFN-γ and IL-4 were very low although both LMWC and oligomixture statistically increased IFN-γ secretion (Fig 4A & 4B).

Both IFN-γ and IL-4 secretion by splenocytes in response to ConA were markedly augmented in all experimental groups (Fig 4A & 4B). The IFN-γ/IL-4 ratio for mice that received with hydrolysate, LMWC, and oligomixture were 2.5 ± 0.3, 5.0 ± 0.1, and 10.2 ± 1.0, respectively, which were significant higher than that of the control (1.3 ± 0.1) (Fig. 4C). These results demonstrated that oral administration of chitosan hydrolytic products would modulate Th1/Th2 immune balance toward Th1 polarization.

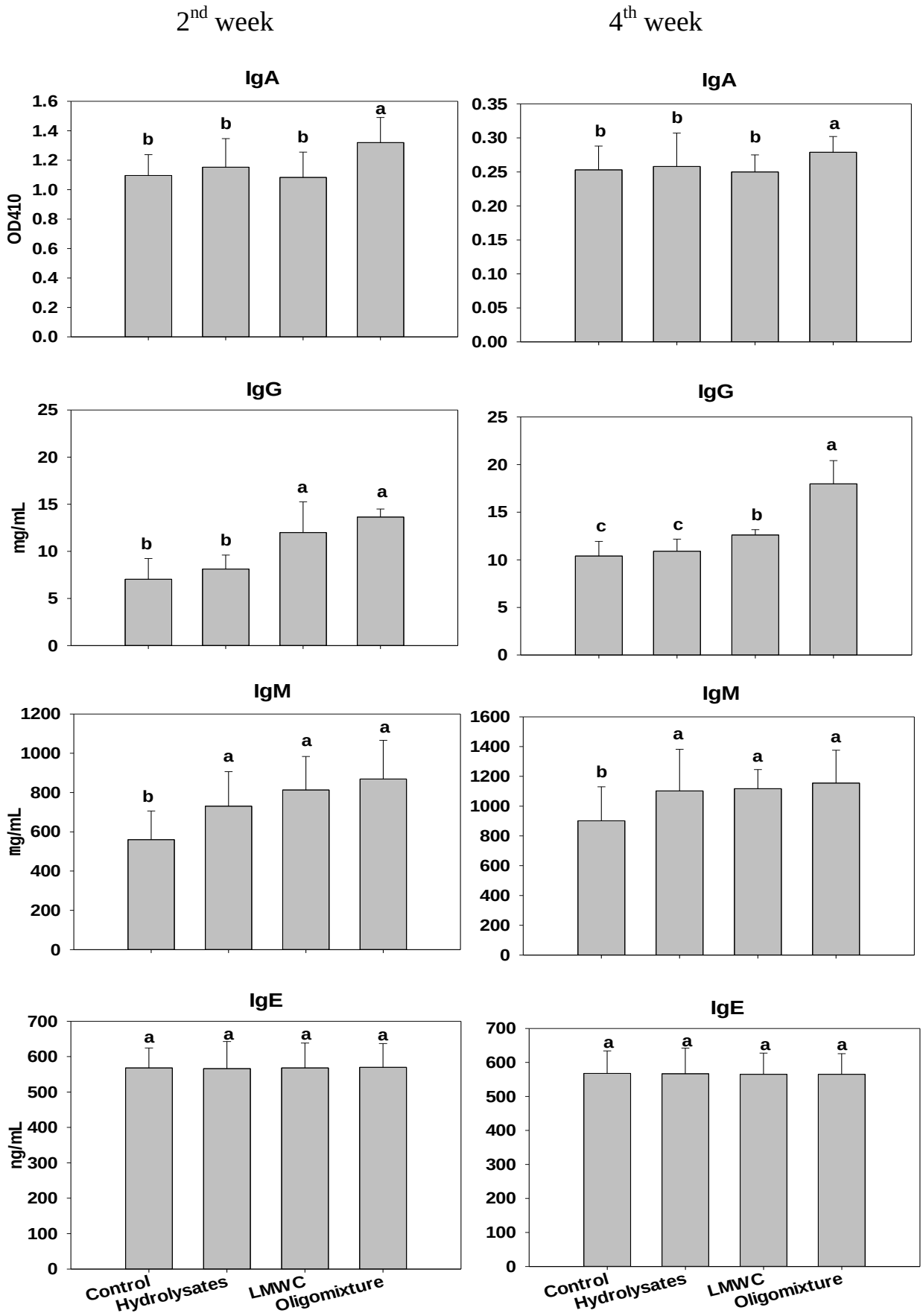
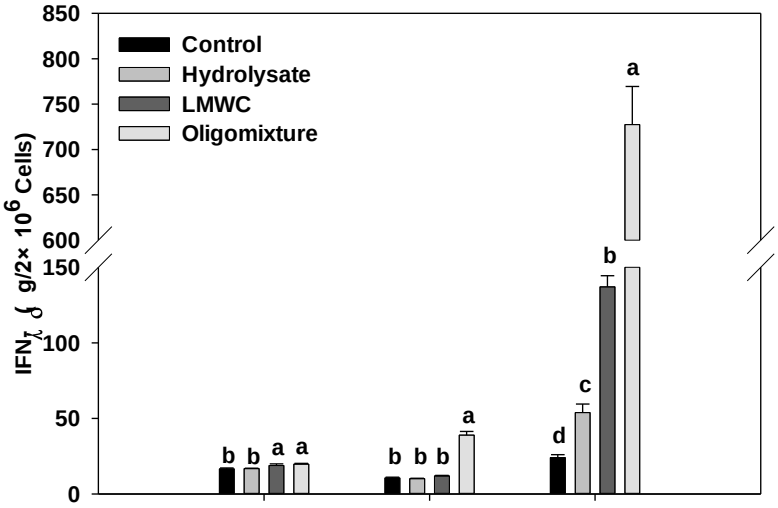


Fig. 3 Chang et al

(A)



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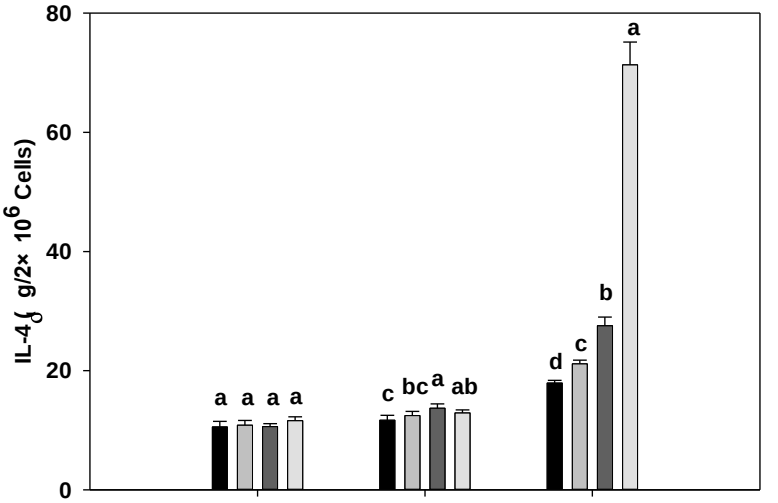

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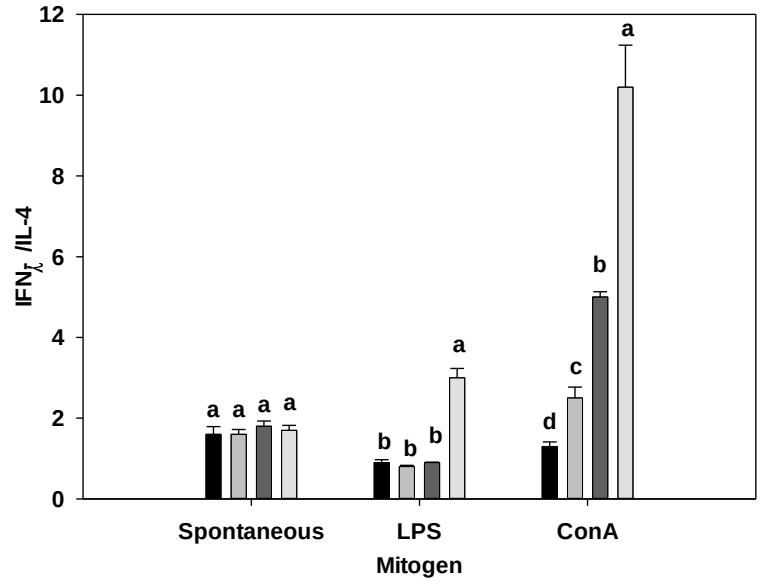


Fig. 4. Chang et al

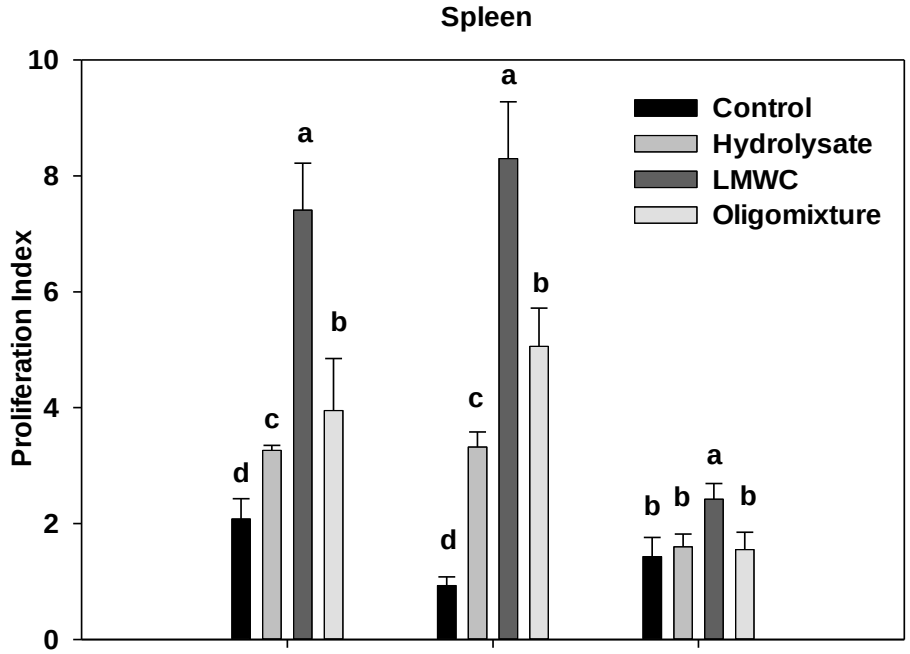
3.2. Oral administration of chitosan hydrolytic products modulated antigen-specific and mitogen-induced immune responses in OVA-immunized mice.

Chitosan has been reported as an effective adjuvant by changing the permeability of epithelia for increasing the uptake of antigens and the contact with the immune system [11, 33]. As the vaccine adjuvant, chitosan could increase cell-mediated immunity and circulating antibody production [9, 28]. Water-soluble chitooligosaccharides and N-acetyl chitooligosaccharides—chitohexaose and *N*-acetyl chitohexaose in particular—have also been considered as immunopotentiators [17, 34]. Although many studies have investigated the modulatory effects of chitosan on antigen-specific immune responses, chitosan was applied as vaccine adjuvants in most of these studies. In the present study, we addressed whether oral administration with chitosan hydrolytic products affects antigen-specific immune responses in OVA-immunized BALB/c mice.

As shown in Fig. 5A, the proliferation index of splenocytes co-cultured in vitro with OVA for the group that received LMWC was significantly higher than that of the control (Fig. 5A). All samples of hydrolysate, LMWC, and oligomixture significantly increased the ConA- and LPS-stimulated splenocyte proliferation in OVA-immunized mice; the LMWC group in particular showed markedly enhanced splenocyte proliferation compared with the control group (Fig. 5A). The in vitro proliferation indexes of PP cells co-cultured with OVA from the groups receiving hydrolysate and oligomixture were significantly higher than that of the control (Fig. 5B). Compared with the proliferation index of PP B cells (stimulated by LPS) without OVA immunization (Fig. 2B), the proliferation of PP B cells markedly increased after OVA immunization (Fig. 5B). Although the reason for the differences in enhancement profiles between the splenocytes and the PP cells is presently unclear, we are confident that oral administration of the chitosan hydrolytic products activates the splenocytes by increasing their proliferation with or without antigen immunization.

The modulatory effects of chitosan hydrolytic products on the OVA-specific humoral immune responses of BALB/c mice were also evaluated (Fig. 6). The OVA-specific IgM (Fig. 6A), IgG (Fig. 6B), and IgA levels (Fig. 6C) were significantly increased for the group that received oligomixture compared to those of the control. The OVA-specific IgE levels were similar for all groups (Fig. 6D). Moreover, the levels of IgG subclasses, including both IgG1 and IgG2a, were also measured. The level of IgG1 in the group that received LMWC was significantly decreased; while the level of IgG2a in the group that received oligomixture was significantly increased **(**Fig. 7**)**.

(A)



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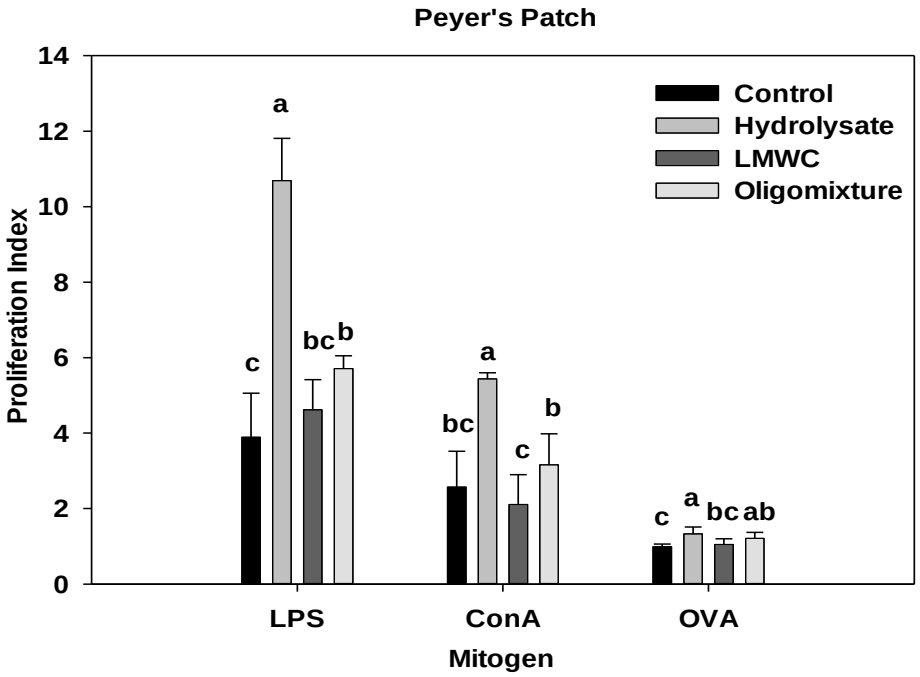


Fig. 5 Chang et al.

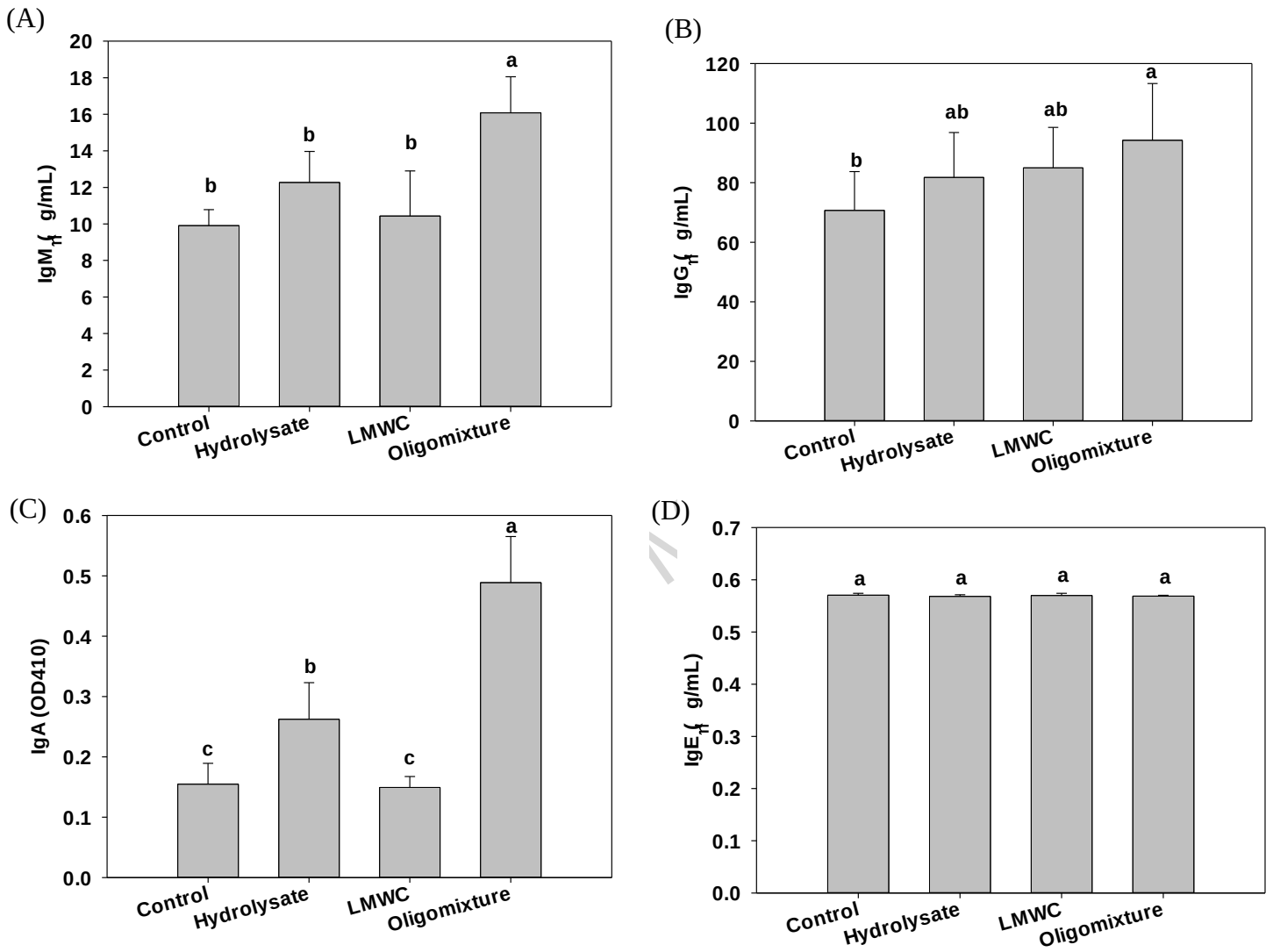
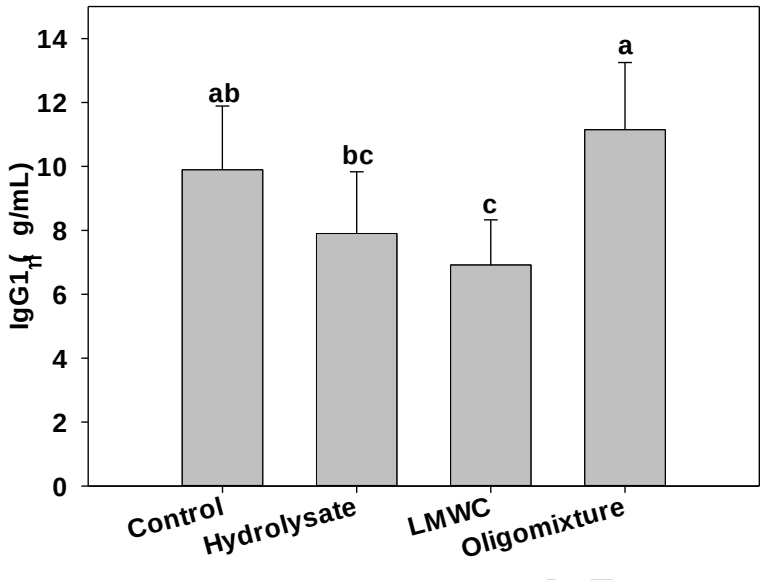


Fig. 6 Chang et al.

(A)



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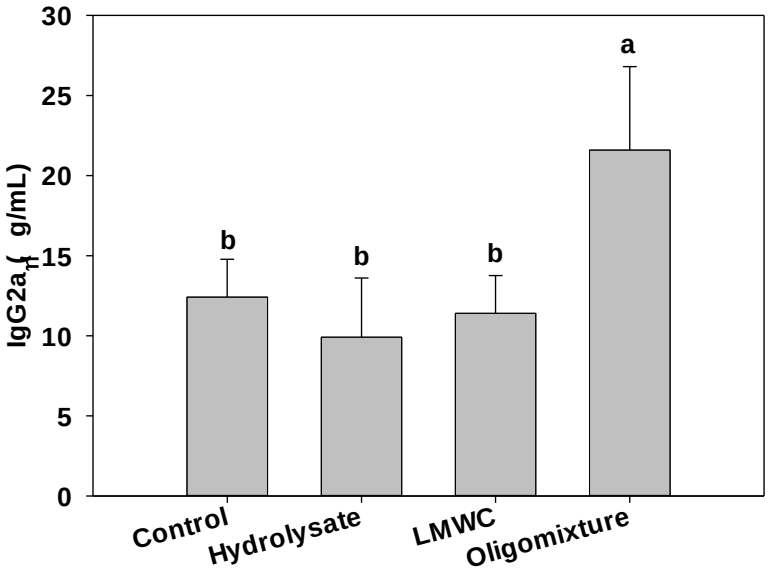

Fig. 7. Chang et al.

Due to the distinct effects of LMWC and oligomixture on modulating IgG1 and IgG2a production, the effects of chitosan hydrolytic products on modulating the secretion of the IFN-γ (Th1 cytokine, promoting IgG2a production) and IL-4 (Th2 cytokine; promoting IgG1 production) were examined. The spontaneous IFN-γ production was significantly increased in mice that received oligomixture **(**Fig. 8A), and the spontaneous IL-4 production was significantly decreased for all experimental groups **(**Fig. 8B**)**.

When splenocytes were co-cultured with OVA or Con A, the IFN-γ secretion was

significantly enhanced in the groups receiving the LMWC and oligomixture, while IL-4 secretion was significantly inhibited for all experimental groups. Accordingly, the IFN-γ/IL-4 ratio was significantly increased for all experimental groups (Figs. 8A, B, C). Cytokines have been reported to affect antibody class switching; for example, IL-4 causes antibody class switch to IgE, IgG1, and IgG4 [35, 36], IFN-γ causes antibody class switch to IgG2a and IgG3 [37, 38] and TGF-β causes the IgA class switch [39]. The significant increase in IFN-γ production and decrease in IL-4 production upon the application of the three chitosan hydrolytic products (Fig. 8) may partly explain the increase in IgG2a in the oligomixture-receiving group and the decrease in IgG1 in the LMWC-receiving group (Fig. 7). Shibata, et al. [40] have shown that oral administration of chitin resulted in decreased IL-4 and increased IFN-γ production. Concordantly, our results show that the immune responses were redirected toward a Th1 response by both LMWC and oligomixture. Chitin and chitosan have been shown to induce the TGF-β production by PP cells [41], macrophages [25], and platelets [42]. Nonetheless, except for TGF-β, numerous factors, such as CD40L, TLR4 ligand, BAFF and APRIL, are also known to induce IgA switch [43]. Further comprehensive investigations are required to elucidate the action mechanism of oligomixture-induced IgA production.

It has been reported that particulate antigens exhibit dissimilar immunologic properties with that to soluble antigens. Particulate antigens tend to be selectively phagocytized by APCs to elicit cellular immune responses. In contrast, soluble antigens tend to be internalized via pinocytosis to explore humoral immune responses [44]. With respect to polysaccharide immunomodulators, particulate β-glucan also showed the capability of inducing Th1 cell activation compared to the soluble one [45]. It is expectable that chitosan hydrolytic products with higher molecular weight are relative insoluble than that with lower molecular weight. Therefore, in the antigen- specific response it is reasonable that oligomixture was more potent to elicit antibody production, while LMWC was more potent to elevate lymphocyte proliferation and IFN-γ secretion. On the other hand, it has been reported that LPS and chitosan share a binding site on monocytes which involves CD14, a co-receptor with TLR4 [46, 47]. Qiao *et al*. have substantiated that chitosan oligosaccharides could inhibit binding of LPS to TLR4 and thus reduce the production of pro-inflammatory mediators [48]. In contrast, Zhang *et al*. have demonstrated that chitosan oligosaccharides possessed immune-stimulating properties by activating TLR4 [49]. Therefore, we suggest that the different impact of

chitosan hydrolytic products on the activation receptor of immune cells may be another potential mechanism for their distinct immunomodulatory properties. The underlying mechanistic relationship between the molecular weight, structure, solubility and binding characteristics of chitosan hydrolytic products with their distinct immunomodulatory properties is an intriguing issue that warrants further investigations.

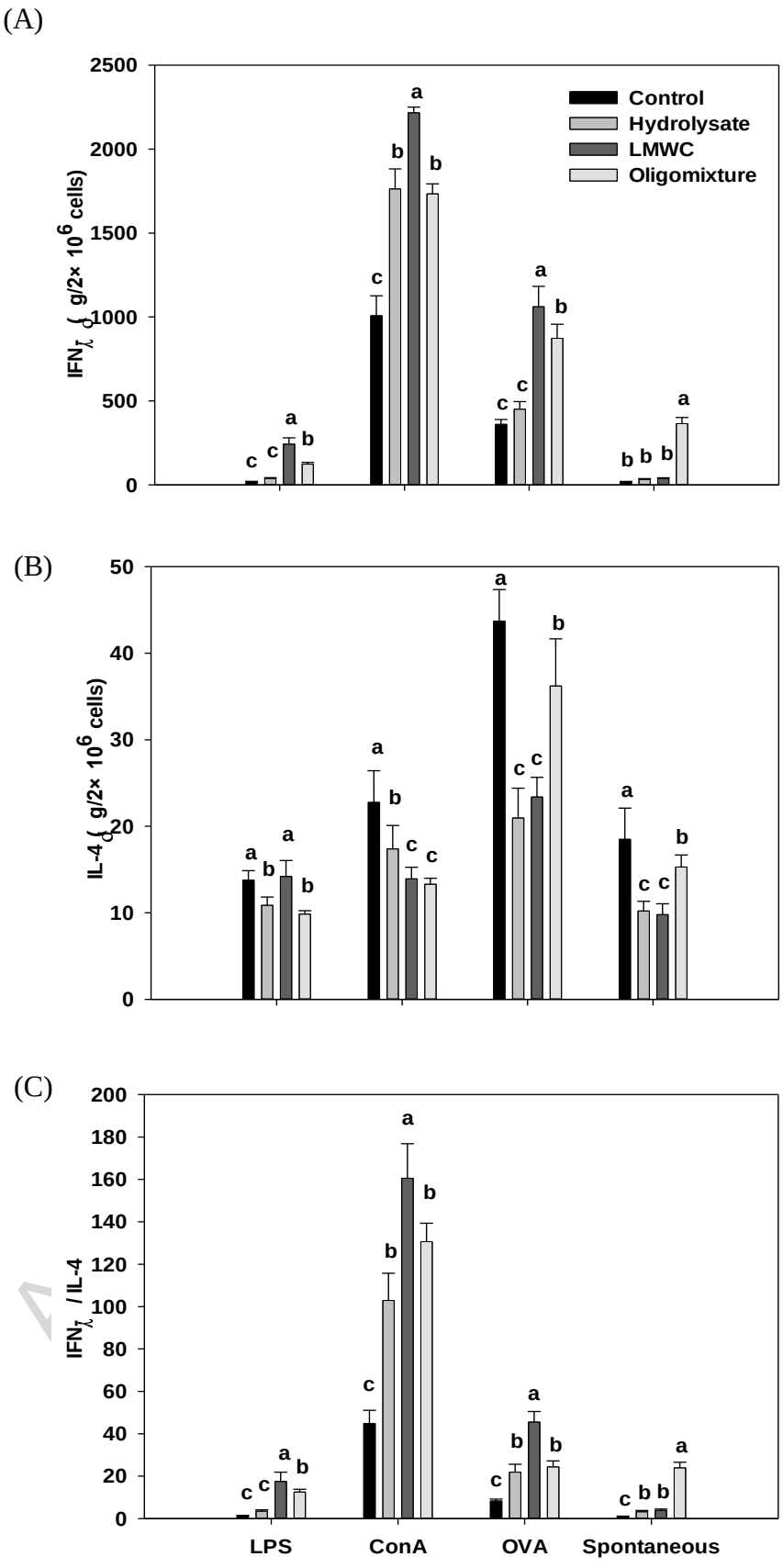

Fig. 8. Chang et al.

In summary, oral administration of the chitosan hydrolytic products of LMWC, and oligomixture modulated mitogen-specific and OVA-specific immune responses. Especially, oligomixture was more potent to elicit OVA-specific antibody production; while LMWC was more potent to elevate splenocytes proliferation and splenic IFN-γ secretion in OVA-immunized mice. Our results provide the distinct immunomodulatory properties of chitosan hydrolytic products in response to mitogens and specific antigen, suggesting that dietary chitosan hydrolytic products may be helpful for the prevention and therapy of immune disorders and pathogen infection.

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References:

[1] B. Pulendran, R. Ahmed, Immunological mechanisms of vaccination, Nat Immunol 12(6) (2011) 509-17.

[2] C. Kantari, M. Pederzoli-Ribeil, V. Witko-Sarsat, The role of neutrophils and monocytes in innate immunity, Contrib Microbiol 15 (2008) 118-46.

[3] M. Locati, A. Mantovani, A. Sica, Macrophage activation and polarization as an adaptive component of innate immunity, Adv Immunol 120 (2013) 163-84.

[4] C. Liongue, L.B. John, A. Ward, Origins of adaptive immunity, Crit Rev Immunol 31(1) (2011) 61-71.

[5] S.C. Corr, C.C. Gahan, C. Hill, M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis, FEMS Immunol Med Microbiol 52(1) (2008) 2-12.

[6] J. Kunisawa, Y. Kurashima, H. Kiyono, Gut-associated lymphoid tissues for the development of oral vaccines, Adv Drug Deliv Rev 64(6) (2012) 523-30.

[7] N.A. Mabbott, D.S. Donaldson, H. Ohno, I.R. Williams, A. Mahajan, Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium, Mucosal Immunol 6(4) (2013) 666-77.

[8] P. Brandtzaeg, Function of mucosa-associated lymphoid tissue in antibody formation, Immunol Invest 39(4-5) (2010) 303-55.

[9] A. Chakrabarti, D. Talukdar, A. Pal, M. Ray, Immunomodulation of macrophages by methylglyoxal conjugated with chitosan nanoparticles against Sarcoma-180 tumor in mice, Cell Immunol 287(1) (2014) 27-35.

[10] D.P. Vasconcelos, A.C. Fonseca, M. Costa, I.F. Amaral, M.A. Barbosa, A.P. Aguas, J.N. Barbosa, Macrophage polarization following chitosan implantation, Biomaterials 34(38) (2013) 9952-9.

[11] J. Feng, L. Zhao, Q. Yu, Receptor-mediated stimulatory effect of oligochitosan in macrophages, Biochem Biophys Res Commun 317(2) (2004) 414-20.

[12] J. Liang, F. Li, Y. Fang, W. Yang, X. An, L. Zhao, Z. Xin, L. Cao, Q. Hu, Cytotoxicity and apoptotic effects of tea polyphenol-loaded chitosan nanoparticles on human hepatoma HepG2 cells, Mater Sci Eng C Mater Biol Appl 36 (2014) 7-13.

[13] G.J. Wu, G.J. Tsai, Chitooligosaccharides in combination with interferon-γ increase nitric oxide production via nuclear factor-κB activation in murine RAW264. 7 macrophages, Food Chem. Toxicol. 45(2) (2007) 250-258.

[14] S.P. Strand, S. Danielsen, B.E. Christensen, K.M. Vårum, Influence of chitosan structure on the formation and stability of DNA− chitosan polyelectrolyte complexes, Biomacromolecules 6(6) (2005) 3357-3366.

[15] B. Zheng, Z.-S. Wen, Y.-J. Huang, M.-S. Xia, X.-W. Xiang, Y.-L. Qu, Molecular weight-dependent immunostimulative activity of low molecular weight chitosan via regulating NF-κB and AP-1 signaling pathways in RAW264. 7 macrophages, Mar

Drugs 14(9) (2016) 169.
[16] S.K. Panda, S. Kumar, N.C. Tupperwar, T. Vaidya, A. George, S. Rath, V. Bal, B. Ravindran, Chitohexaose activates macrophages by alternate pathway through TLR4 and blocks endotoxemia, PLoS Pathog 8(5) (2012) e1002717.
[17] K. Suzuki, A. Tokoro, Y. Okawa, S. Suzuki, M. Suzuki, Effect of N-acetylchito-oligosaccharides on activation of phagocytes, Microbiol Immunol 30(8) (1986) 777-87.
[18] G.-J. Wu, G.-J. Tsai, Cellulase degradation of shrimp chitosan for the preparation of a water-soluble hydrolysate with immunoactivity, Fish Sci 70(6) (2004) 1113-1120.
[19] G.J. Tsai, W.H. Su, Antibacterial activity of shrimp chitosan against *Escherichia coli*, J Food Prot 62(3) (1999) 239-43.
[20] S.H. Chang, H.T. Lin, G.J. Wu, G.J. Tsai, pH Effects on solubility, zeta potential, and correlation between antibacterial activity and molecular weight of chitosan, Carbohydr Polym 134 (2015) 74-81.
[21] M.B. Hansen, S.E. Nielsen, K. Berg, Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill, J Immunol Methods 119(2) (1989) 203-10.
[22] J.G. Lewis, D.O. Adams, S. Fan, Selective sensitivity of macrophages to cytotoxicity by inhibitors of macromolecular synthesis: induction of apoptosis, J Leukoc Biol 57(4) (1995) 635-42.
[23] B. Bin-Hafeez, R. Haque, S. Parvez, S. Pandey, I. Sayeed, S. Raisuddin, Immunomodulatory effects of fenugreek (*Trigonella foenum graecum L.*) extract in mice, Int Immunopharmacol 3(2) (2003) 257-65.
[24] S.P. James, M. Zeitz, Human gastrointestinal mucosal T cells, Handbook of mucosal immunology, Academic Press, San Diego, 1994, pp. 275-285.
[25] C. Porporatto, I.D. Bianco, S.G. Correa, Local and systemic activity of the polysaccharide chitosan at lymphoid tissues after oral administration, J Leukoc Biol 78(1) (2005) 62-9.
[26] Y. Maeda, Y. Kimura, Antitumor effects of various low-molecular-weight chitosans are due to increased natural killer activity of intestinal intraepithelial lymphocytes in sarcoma 180–bearing mice, J. Nutr. 134(4) (2004) 945-950.
[27] T. Diamantstein, M. Klos, H. Osawa, Z. Chen, Chitin: an immunological adjuvant and a polyclonal B-lymphocyte activator, Int Arch Allergy Immunol 68(4) (1982) 377-381.
[28] X. Li, M. Min, N. Du, Y. Gu, T. Hode, M. Naylor, D. Chen, R.E. Nordquist, W.R. Chen, Chitin, chitosan, and glycated chitosan regulate immune responses: the novel adjuvants for cancer vaccine, Clin Dev Immunol 2013 (2013) 387023.
[29] B.S. Powell, A.K. Andrianov, P.C. Fusco, Polyionic vaccine adjuvants: another

look at aluminum salts and polyelectrolytes, Clin Exp Vaccine Res 4(1) (2015) 23-45.
[30] Z.S. Wen, Y.L. Xu, X.T. Zou, Z.R. Xu, Chitosan nanoparticles act as an adjuvant to promote both Th1 and Th2 immune responses induced by ovalbumin in mice, Mar Drugs 9(6) (2011) 1038-55.
[31] S.H. Lee, E.A. Bae, E.K. Park, Y.W. Shin, N.I. Baek, E.J. Han, H.G. Chung, D.H. Kim, Inhibitory effect of eupatilin and jaceosidin isolated from Artemisia princeps in IgE-induced hypersensitivity, Int Immunopharmacol 7(13) (2007) 1678-84.
[32] S.L. Swain, L.M. Bradley, M. Croft, S. Tonkonogy, G. Atkins, A.D. Weinberg, D.D. Duncan, S.M. Hedrick, R.W. Dutton, G. Huston, Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development, Immunol Rev 123 (1991) 115-44.
[33] M. Thanou, J.C. Verhoef, H.E. Junginger, Oral drug absorption enhancement by chitosan and its derivatives, Adv Drug Deliv Rev 52(2) (2001) 117-26.
[34] W.G. Seo, H.O. Pae, N.Y. Kim, G.S. Oh, I.S. Park, Y.H. Kim, Y.M. Kim, Y.H. Lee, C.D. Jun, H.T. Chung, Synergistic cooperation between water-soluble chitosan oligomers and interferon-gamma for induction of nitric oxide synthesis and tumoricidal activity in murine peritoneal macrophages, Cancer Lett 159(2) (2000) 189-95.
[35] M.T. Berton, J.W. Uhr, E.S. Vitetta, Synthesis of germ-line gamma 1 immunoglobulin heavy-chain transcripts in resting B cells: induction by interleukin 4 and inhibition by interferon gamma, Proc Natl Acad Sci U S A 86(8) (1989) 2829-33.
[36] H. Gascan, J.F. Gauchat, M.G. Roncarolo, H. Yssel, H. Spits, J.E. de Vries, Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated CD4+ T cell clones, J Exp Med 173(3) (1991) 747-50.
[37] J.T. Collins, W.A. Dunnick, Germline transcripts of the murine immunoglobulin gamma 2a gene: structure and induction by IFN-gamma, Int Immunol 5(8) (1993) 885-91.
[38] P. Zelazowski, J.T. Collins, W. Dunnick, C.M. Snapper, Antigen receptor cross-linking differentially regulates germ-line CH ribonucleic acid expression in murine B cells, J. Immunol. 154(3) (1995) 1223-1231.
[39] P. Shockett, J. Stavnezer, Effect of cytokines on switching to IgA and alpha germline transcripts in the B lymphoma I.29 mu. Transforming growth factor-beta activates transcription of the unrearranged C alpha gene, J Immunol 147(12) (1991) 4374-83.
[40] Y. Shibata, L.A. Foster, J.F. Bradfield, Q.N. Myrvik, Oral administration of chitin down-regulates serum IgE levels and lung eosinophilia in the allergic mouse, J Immunol 164(3) (2000) 1314-21.

[41] M.I. Oliveira, S.G. Santos, M.J. Oliveira, A.L. Torres, M.A. Barbosa, Chitosan drives anti-inflammatory macrophage polarisation and pro-inflammatory dendritic cell stimulation, Eur Cell Mater 24 (2012) 136-52; discussion 152-3.

[42] Y. Okamoto, R. Yano, K. Miyatake, I. Tomohiro, Y. Shigemasa, S. Minami, Effects of chitin and chitosan on blood coagulation, Carbohydr Polym 53(3) (2003) 337-342.

[43] A. Cerutti, The regulation of IgA class switching, Nature reviews immunology 8(6) (2008) 421.

[44] C.M. Snapper, Distinct Immunologic Properties of Soluble Versus Particulate Antigens, Front Immunol 9 (2018) 598.

[45] C. Qi, Y. Cai, L. Gunn, C. Ding, B. Li, G. Kloecker, K. Qian, J. Vasilakos, S. Saijo, Y. Iwakura, Differential pathways regulating innate and adaptive anti-tumor immune responses by particulate and soluble yeast-derived β-glucans, Blood 117(25) (2011) 6825-6836.

[46] H.J. Shin, H. Lee, J.D. Park, H.C. Hyun, H.O. Sohn, D.W. Lee, Y.S. Kim, Kinetics of binding of LPS to recombinant CD14, TLR4, and MD-2 proteins, Molecules & Cells (Springer Science & Business Media BV) 24(1) (2007).

[47] M. Otterlei, K.M. Vårum, L. Ryan, T. Espevik, Characterization of binding and TNF-α-inducing ability of chitosans on monocytes: the involvement of CD14, Vaccine 12(9) (1994) 825-832.

[48] Y. Qiao, Y. Ruan, C. Xiong, Q. Xu, P. Wei, P. Ma, X. Bai, Y. Du, Chitosan oligosaccharides suppressant LPS binding to TLR4/MD-2 receptor complex, Carbohydrate Polymers 82(2) (2010) 405-411.

[49] P. Zhang, W. Liu, Y. Peng, B. Han, Y. Yang, Toll like receptor 4 (TLR4) mediates the stimulating activities of chitosan oligosaccharide on macrophages, Int Immunopharmacol 23(1) (2014) 254-261.

Figure captions

Fig. 1. Effects of oral administration of chitosan hydrolysate, LMWC, and oligomixture on the body weight (A) and phagocytic activity of peritoneal macrophages (B) of BALB/c mice. The mice were divided into four groups (10 mice per group) and administrated orally with 0.5 ml saline (for control group), chitosan hydrolysate, LMWC, or oligomixture at the dosage of 500 mg/kg body weight (for experimental group) 6 times per week for 4 weeks by gavage. (A) The body weight of each mouse was recorded per week. (B) After 4 weeks of treatment, the mice were sacrificed, and the peritoneal macrophages were harvested for the assay of phagocytic activity as described in the **Materials and methods**. Data are expressed as mean with SD (n=10). Differences of $p < 0.05$ were considered statistically significant.

Fig. 2. Effects of oral administration of chitosan hydrolysate, LMWC, and oligomixture on mitogen-stimulated proliferation of splenocytes (A) and PP (B) lymphocytes. The mice were treated as described in the **Materials and methods.** After 4 weeks of treatment, the mice were sacrificed, and splenocytes and Peyer's patch (PP) lymphocytes were prepared and cultured in the absence or presence of LPS or ConA for 48 h. Cell proliferation was determined by MTT assay. Data are expressed as mean with SD (n=10). Differences of $p < 0.05$ were considered statistically significant.

Fig. 3. Effects of oral administration of chitosan hydrolysate, LMWC, and oligomixture on the levels of IgA(A), IgG(B), IgM(C), and IgE(D) production in serum of BALB/c mice. The mice were treated as described in the **Materials and methods.** After 2 and 4 weeks of treatment, the serum samples were collected, and the levels of total IgA, IgG, IgM, and IgE production in sera were determined by ELISA. Data are expressed as mean with SD (n=10). Differences of $p < 0.05$ were considered statistically significant.

Fig. 4. Effects of oral administration of chitosan hydrolysate, LMWC, and oligomixture on the secretion of IFN-γ (A) and IL-4 (B), and IFN-γ/IL-4 ratio by splenocytes. The mice were treated as described in the **Materials and methods.** After 4 weeks of treatment, the mice were sacrificed, and splenocytes were prepared and cultured in the absence or presence of LPS or ConA. After 48

h, the levels of IFN-γ and IL-4 secretion in the supernatants were determined by ELISA. Further, the ratio of IFN-γ/IL-4 was evaluated. Data are expressed as mean with SD (n=10). Differences of $p < 0.05$ were considered statistically significant.

Fig. 5. Effects of oral administration of chitosan hydrolysate, LMWC, and oligomixture on mitogen- or OVA-induced proliferation of splenocytes (A) and PP (B) lymphocytes harvested from OVA-immunized mice. The mice were treated as described in the **Materials and methods.** After 4 weeks of treatment, the mice were sacrificed, and splenocytes and Peyer's patch (PP) lymphocytes were prepared and cultured in the presence of OVA, LPS or ConA for 48 h. Cell proliferation index was determined by MTT assay. Data are expressed as mean with SD (n=10). Differences of $p < 0.05$ were considered statistically significant.

Fig. 6. Effects of oral administration of chitosan hydrolysate, LMWC, and oligomixture on the levels of OVA-specific IgM(A), IgG(B), IgA(C), and IgE(D) in sera of OVA-immunized BALB/c mice. OVA-immunized mice were treated as described in the **Materials and methods.** After 4 weeks of treatment, the serum samples were collected, and the levels of OVA-specific IgA, IgG, IgM, and IgE production in sera were determined by ELISA. Data are expressed as mean with SD (n=10). Differences of $p < 0.05$ were considered statistically significant.

Fig. 7. Effects of oral administration of chitosan hydrolysate, LMWC, and oligomixture on the levels of OVA-specific IgG1(A) and IgG2a(B) in sera of OVA-immunized BALB/c mice. OVA-immunized mice were treated as described in the **Materials and methods.** After 4 weeks of treatment, the serum samples were collected, and the levels of OVA-specific IgG1 and IgG2a production in sera were determined by ELISA. Data are expressed as mean with SD (n=10). Differences of $p < 0.05$ were considered statistically significant.

Fig. 8. Effects of oral administration of chitosan hydrolysate, LMWC, and oligomixture on the secretion of IFN-γ (A) and IL-4 (B), and IFN-γ/IL-4 ratio by splenocytes harvested from OVA-immunized BALB/c mice. OVA-immunized mice were treated as described in the **Materials and methods.** After 4 weeks of treatment, the mice were sacrificed, and splenocytes were

prepared and cultured in the absence or presence of OVA, LPS or ConA. After 48 h, the levels of IFN-γ and IL-4 secretion in the supernatants were determined by ELISA. Further, the ratio of IFN-γ/IL-4 was evaluated. Data are expressed as mean with SD (n=10). Differences of $p < 0.05$ were considered statistically significant.