IMMUNOMODULATORY ACTIVITY OF ORAL ADMINISTRATION OF THE CHITOSAN HYDROLYTIC PRODUCTS IN BALB/C MICE

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ABSTRACT

A water-soluble chitosan hydrolysate was obtained by cellulase degradation of chitosan, and a low-molecular-weight chitosan (LMWC) and chitooligosaccharide mixtire (oligomixture) were separated from this hydrolysate. The aim of this study was to evaluate the non-specific and ovalbumin (OVA)-specific immunomodulatory activities of these chitosan hydrolytic products. After oral administration of BALB/c mice with chitosan hydrolysate, LMWC and oligomixture at the dose of 500 mg/kg for 4 weeks, the phagocytic activity of mouse peritoneal macrophages was significantly increased in hydrolysate- and oligomixture- feeding groups. All of the chitosan hydrolytic products significantly increased concanavalin A (ConA)-stimulated proliferation of splenocytes and Peyer's patch lymphocytes, and also lipopolysaccharide (LPS)-stimulated splenocytes proliferation. The serum IgG and IgM levels were significantly higher in mice fed with LWMC or oligomixture than those of the control group. LMWC and oligomixture also up-regulated IFN-γ secretion of splenocytes, and induced predominantly Th1 cytokine secretion in splenocytes. In OVA-specific immunomodulation, oligomixture and LMWC significantly increased ConA- or LPS- stimulated proliferation of splenocytes; while only oligomixture increased mitogen-stimulated proliferation of Peyer's patch lymphocytes. Amounts of OVA-specific serum antibodies of IgM, IgG, and IgA, but not IgE, were significantly enhanced by oligomixture. All of chitosan hydrolytic products significantly augmented IFN-γ/IL-4 (Th1/Th2) ratio. These observations indicate that the chitosan hydrolytic products stimulate splenocytes to polarize Th1 type responses in BALB/c mice.

Keywords: chitosan hydrolysate; chitooligosaccharides; Peyer's patch; macrophages; Th1; immunomodulation.

INTRODUCTION

Chitin (poly-β-(1→4) *N*-acetyl-D-glucosamine) and chitosan (a partially or fully deacetylated chitin) have various physiological activities. They can accelerate wound healing in animals [1], activate macrophages, complement proteins and cytotoxic T lymphocytes [2,3] and protect animals from pathogen infections [4,5]. However, the water-insolubility of chitin and chitosan make them disadvantageous as immunotherapeutic agents. The water-soluble chitooligosaccharides and *N*-acetyl chitooligosaccharides, in particular, the hexamers of *N*-acetyl chitohexaose and chitohexaose, have recently attracted much attention for their immune-enchancing effects [6,7] and antitumor activity [8,9]. However, the very high cost of these hexamers limits their application. A water-soluble chitosanolytic product with immunoactivity merits developed.

Although various acids, such as HCl, HF, phosphoric acid and nitrous acid, have been used to hydrolyze chitin or chitosan [10-14], enzymatic digestion becomes more

popular due to its mild reaction condition and good reproduction [15-17]. In our previous study a water-soluble chitosan hydrolysate with immunomodulation activity was obtained by cellulase degradation of chitosan [18]. A low-molecular-weight chitosan (LMWC) and a chitooligosaccharide mixture isolated from this chitosan hydrolysate were shown to have different stimulatory effects on the cell proliferation and IgM secretion of the human hybridoma HB4C5 cells [18]. This chitooligosaccharide mixture can increase the phagocytic activity and nitric oxide (NO) secretion in INF-γ –primed murine 264.7 macrophages [19]. In this study the immunomodulatory activities of these chitosan hydrolytic products on non-specific and OVA-specific immune responses were evaluated by oral administration in BALB/c mice.

EXPERIMENTs

Production of chitosan hydrolysate and component separation. According to the protocol of Wu and Tsai [18], in a 5-L-fermentor (CMCF-5, Chin Chi Co., Taipei, ROC) containing 2000 mL acetic acid- bicarbonate buffer (pH 5.2) 100 g chitosan with 95% deacetylation were added, followed by the addition of cellulase (10 U/mL). After digestion at 55°C with 125 rpm for 9 h the hydrolysate was neutralized and centrifuged (12000 ×*g*, 30 min). The supernatant (designated as chitosan hydrolysate in this study) was added with an equal volume of methanol and fractionated into two fractions: the upper aliquot chitooligosaccharide mixture (oligomixture) and a low-molecular-weight chitosan (LMWC) in the precipitate. All samples were lyophilized and stored at 25°C before use.

Experimental design. The 5- week old mice were divided into four groups, each containing ten mice, and were administrated orally with 0.5 mL saline, chitosan hydrolysate, LMWC, or oligomixture at the dosage of 500 mg/kg body weight 6 times per week for 4 weeks by gastric tube. Sera were collected on the 2^{nd} and 4^{th} weeks for measurement of total antibody by ELISA [18]. Animals were then sacrificed by cervical dislocation and peritoneal macrophages were collected. The splenocytes and Peyer's patch lymphocytes were collected for proliferation assay.

For specific immunomodulation studies, mice were immunized by an intraperitoneal injection of 0.2 mL OVA solution (50 μg/mL) on the 2^{nd} week. A boosting injection was given on the 4^{th} week. Splenocytes and Peyer's patch cells were collected 1 week after t he second immunization for proliferation assay. Sera were collected on the 2^{nd}, 3^{th}, and 5^{th} weeks for measurement of OVA-specific antibody.

Phagocytosis of peritoneal macrophage. The peritoneal macrophages (1×10^6 cells/mL) were incubated with *Saccharomyces cervisiae* CCRC21607 cells (1×10^7 cells/mL), which were pre-incubated in 10% FCS at 37°C for 30 min, at 37°C for 1 h. The phagocytosis of macrophages was then microscopically assessed after H&E staining.

Lymphocyte proliferation assay. One hundred μL of the spleen or Peyer's patch lymphocyte suspension (2×10^6 cells/mL) were placed on a 96-well microplate, and followed by the addition of 20 μL of lipopolysaccharide (LPS, 30 μg/mL, Sigma) or concanavalin A (Con A, 20 μg/mL, Sigma) in triplicate. After incubation at 37 ^{o}C for 48 h, the viable cells were measured by MTT assay [20]. Lymphocyte proliferation

(%) was calculated as follows: proliferation rate (%) = [$Exp._{ConA\ or\ LPS}$ / T] ×100, where $Exp._{ConA\ or\ LPS}$ is the optical density of sample well containing ConA or LPS; T is that of control well without ConA or LPS.

Determination of cytokines by ELISA. The amounts of IFN-γ and IL-4 in splenocyte supernatant were quantified using the respective DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN, USA) as directed by the manufacturer.

RESULTS AND DISCUSSION

During the 4 weeks of oral administration experiment, the body weights for all mice were slightly increased, and the body weight gains were not significantly different among the experimental groups and the control. After feeding of chitosan hydrolysate, LMWC, or oligomixture for 4 weeks, the phagocytic rates of the peritoneal macrophages of mice fed with chitosan hydrolysate, LMWC, or oligomixture were 20.6 ± 5.9%, 8.1 ± 4.0%, and 14.5 ± 4.1%, respectively; while the control group was 6.1 ± 1.6%. Macrophages, acting as phagocytic, microbicidal and tumoricidal cells, are the critical role in the primary line of defense against infections. In previous study the chitosan hydrolysate and its component of oligomixture had been demonstrated to enhance in vitro the NO production in IFN-γ-treated murine RAW264.7macrophages [19]. In this study the chitosan hydrolysate and oligomixture were further proved to enhance in vivo the phagocytic activity of peritoneal macrophages in mice; while LMWC did not have these functions in these two studies.

As well as innate leukocyte responses (phagocytosis by peritoneal macrophages), we also investigated in vivo the lymphoproliferative responses of splenocytes and Peyer's patch lymphocytes. All samples of chitosan hydrolysate, LMWC, and oligomixture significantly increased the mitogen-induced proliferation index of splenocytes (Fig. 1A). All samples of the chitosan hydrolytic products also significantly increased proliferation index of Peyer's patch T-cells (Fig. 1B).

Antibody production represents an end-point affecting response of T lymphocyte-mediated antigen recognition. After 4 weeks of feeding, oligomixture significantly increased serum total IgA, IgG and IgM. LMWC significantly increased total IgG and IgM; while hydrolysate significantly increased IgA level. The IgE level was not affected at all by the tested samples.

In general, naive T cells recognize antigen in the context of MHC molecules via TCR complex and develop into armed effect T cells which can largely be divided into Th1 and Th2 cells [21]. To investigate the effect of chitosan hydrolytic products on the production of IFN-γ and IL-4, which are the main represented cytokines for Th1 and Th2, respectively, the splenocytes were cultured with or without mitogen for 48 h, and the supernatants were harvested. The cytokine levels in supernatants were measured by ELISA. The production of spontaneous IFN-γ, but not IL-4, was significantly increased in mice fed with LMWC and oligomixture. Further, both IFN-γ and IL-4 secretion by splenocytes co-cultured with ConA were markedly augmented in all experimental groups. The IFN-γ/IL-4 ratio of hydrolysate, LMWC, and oligomixture groups were 2.5 ± 0.3, 5.0 ± 0.1, and 10.2 ± 1.0, respectively, which were significant higher than that of control (1.3 ± 0.1). These results show that oral administration of chitosan hydrolytic products modulates immune to polarize Th1-type in mice.

For the OVA-specific immunomodulatory oral feeding test, all samples of

hydrolysate, LMWC, and oligomixture significantly increased the ConA- and LPS-stimulated splenocyte proliferation in OVA-immunized mice; especially LMWC group markedly enhanced the splenocyte proliferation, as comparison with control group. Hydrolysate and oligomixture also significantly increased ConA- and LPS-stimulated Peyer's patch cells proliferation. Compared with the proliferation index of Peyer's patch B cells without OVA immunization, the proliferation of Peyer's patch B cells (stimulated by LPS) were markedly increased after OVA immunization.

The OVA-specific IgM, IgG, and IgA levels, but not IgE, were significantly increased in mice fed with oligomixture. It is suggested that oligomixture regulates the converting of Ig class to IgG and IgA in BALB/c mice immunized with OVA. LMWC significantly decreased OVA-specific serum IgG1 levels, and oligomixture significantly increased OVA-specific serum IgG2a levels in mice immunized with OVA. Further, the production of spontaneous IFN-γ was significantly increased in mice fed with oligomixture, and the spontaneous IL-4 was significantly decreased in all groups. When splenocytes were co-cultured with OVA, the IFN-γ secretion was significantly enhanced in LMWC and oligomixture groups; while the IL-4 secretion was significantly inhibited in all experimental groups. Accordingly, the IFN-γ/IL-4 ratio was significantly increased in all experimental groups.

In summary, the results presented here demonstrated that oral administration of hydrolysate and oligomixture enhanced the phagocytic activity of the peritoneal macrophages. The oligomixture can up-regulate OVA-specific serum IgM, IgG, IgG2a, and IgA, but not IgE, in mice immunized with OVA. The down-regulation of OVA-specific serum IgG1 was obtained in OVA-immunized mice fed with LMWC. Oral administration of chitosan hydrolytic products cause Th1-polarized responses by splenocytes, as characterized by augmenting IFN-γ/IL-4 (Th1/Th2) ratio.

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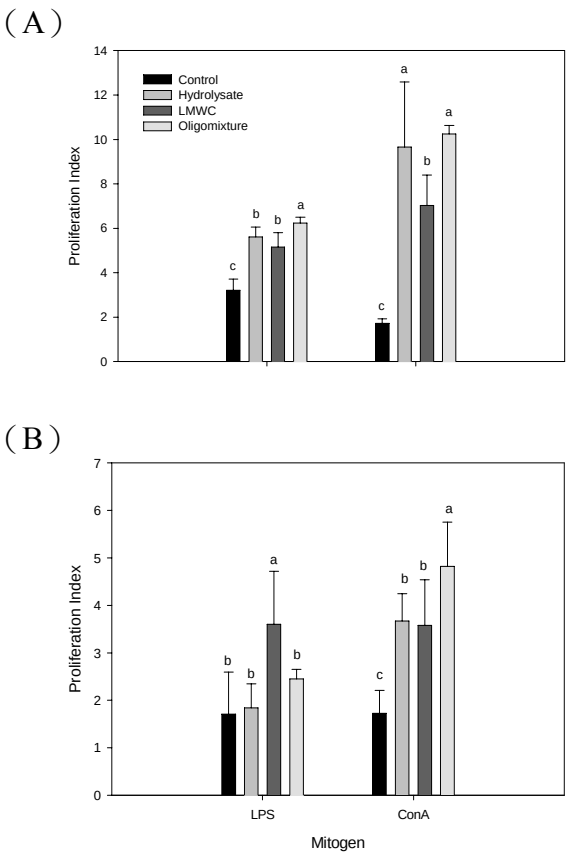

Fig.1 Effects of oral administration of chitosan hydrolysate, LMWC, and oligomixture on mitogen-stimulated proliferation of spleenic (A) and Peyer's patch (B) lymphocytes.