IN VIVO STIMULATION OF PERITONEAL CELLS BY CHITOSAN ADMINISTERED IN DRINKING WATER TO MICE

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Abstract
The aim of the study was to determine immunostimulant properties of chitosan administered alimentary to BALB/c mice. We observed that chitosan feeding effected in activation of cells from the peritoneal cavity. The cells produced less nitric oxide with simultaneous enhanced activity of arginase and higher expression of receptor for IL-4. What is more, chitosan caused increased number of cells expressing MHC class II. The study confirms that chitosan can stimulate immune system what potentially makes it useful candidate for adjuvant.

Key words: *chitosan, innate immunity, adjuvant, macrophages.*

1. Introduction

Protection against various pathogens relies strongly on the action of cells of the innate immunity. Cells initiating an immune response, like granulocytes, natural killer cells, dentritic cells and macrophages, fight the pathogens on the non-specific manner and attract other cells, also of adaptive immunity [1].

Effective vaccination depends not only on the choice of antigen but also the additive substances, particularly adjuvants. They consist of non-specific immune stimulators that enhance vaccine efficacy by boosting immune response, mainly the innate mechanisms [2]. Unfortunaltely, many of them show serious side effects that limit their use. Therefore new substances with immunostimulant activities need to be selected. The perfect adjuvant should be biodegradable and non-toxic while eliciting strong immune response to the co-administered antigen. Preferably it should polarise the Th1 or Th2 type of immune response towards the most effective [3].

Immunostimulant activity of chitosan have been reported repeatedly in different models. Both after subcutaneous [4] and alimentary administration [5], chitosan is able to enhance the immune responses. Increase in cytokine production or membrane receptors expression can be observed. When delivered along with antigen it evokes specific humoral and cellular responses [4]. What is more, it was observed that responses evoked by chitosan may be skewed towards Th2 type [5]. Altogether these properties, among with mucoadhesivity and viscosity, make chitosan appropriate candidate for vaccine adjuvant, particularly for mucosal administration. Yet, the activity of cells in systemic response after oral administration of chitosan has not been reported.

The aim of the study was to determine the phenotype and activity of cells isolated from peritoneal cavity in mice after oral administration of chitosan. Those cells, most abundant source of macrophages, were isolated to estimate nitric oxide production, activity of arginase and expression of surface markers.

2. Materials and methods

2.1 Chitosan

Chitosan of high molecular weight ((310 to >375 kDa) with degree of deacetylation > 75% was obtained from Sigma. 1% stock solution was prepared in 1% acetic acid that was later diluted and administered to mice.

2.2 Mice

Experiment was carried out on male BALB/c mice at the age of 6-8 weeks, weighting 20 - 22 g at the beginning of the study. Animals were given chitosan by alimentary route in drinking water at a dose of 500 μg/day/mouse basing on the water intake. After 10 days of chitosan treatment mice were euthanized.

2.3 Isolation of cells

Cells were isolated from peritoneal cavity by standard method. Mice were injected to the peritoneum with 5 ml of RPMI 1640 supplemented with penicillin/streptomycin (100 μg/ml) and 2 mM L -glutamine (Gibco, UK) and after 1 min of massage peritoneal fluid was collected on ice. Cells were washed sterile three times with fresh RPMI and centrifuged at 250 g for 10 min at 4 ºC. Cells were counted and samples with viability > 97% (*via* Trypan Blue exclusion) were adjusted to concentration of 2.5×10^6 cells/ml with cold RPMI.

2.4 Nitric oxide production and arginase activity assays

Cells were seeded onto 96 well culture plate (Costar, USA), 0.5×10^6 cells per well, and allowed to adhere for 2 h in the incubator (37 ºC, 5% CO_2). Then, non-adherent cells were washed with warm RPMI and the remaining cells (mainly macrophages) were stimulated with 200 μl of medium supplemented with LPS (1 μg/ml), chitosan (5 μg/ml) or medium alone. After 48 h of culture amount of nitric oxide (NO) secreted to the medium was measured with Griess reaction [6], adjusted to 96 well format. Briefly, 100 μl of culture medium were transferred to the 100 μl of Griess reagent and the absorbance was measured after 15 min in microplate spectrometer (μQuant, BioTek, USA) at $\lambda = 550$ nm. Concentration was compared to the standard curve. Cells remained after culture were washed with warm PBS (pH = 7.2) and used to estimate arginase activity as described previously [7], with modifications. Cells on culture plate were lysed with 0.1% Triton X for 1 h on ice and released enzyme was activated with $MnCl_2$ for 15 min at 56 ºC. Then L-arginine was added, the reaction was held for 1 h at 37 ºC and terminated with H_3PO_4, H_2SO_4, H_2O (all reagents from Sigma, Germany). Obtained urea, converted from L-arginine by arginase, was visualised by addition of 9% isonitrosopropiophenone and incubated for 1 h at 95 ºC in the dark. Optical density of coloured complex of urea and isonitrosopropiophenone was measured at $\lambda = 540$ nm.

2.5 Flow cytometry analysis

Isolated peritoneal cells (1×10^6) were re-suspended in PBS and antibodies against MHC class II (conjugated with FITC), IL-4R (conjugated with PE) and MAC1 (conjugated with APC) were added (all antibodies from BD Biosciences, USA). After 30 min incubation on ice, cells were pelleted to wash away unbound antibodies and analysed on FACS Calibur cytometer (BD Biosciences, USA). Cells were gated on monocyte population and percentages of MHC class II and IL4R positive cells were estimated on MAC1+ population.

3. Results and discussion

Peritoneal cells, especially peritoneal macrophages, depending on the activation factor may produce NO or have elevated arginase activity and also may act as antigen presenting cells. They present antigens through MHC class II molecules to T helper cells to activate them. Cytokines that control macrophage activation are: IFNγ and IL-4 which activate cells towards different phenotypes [8].

Various groups reported immunostimulant activity of chitosan. Proporatto et al. [5] showed that rats after feeding with chitosan demonstrated increased production of IL-10,

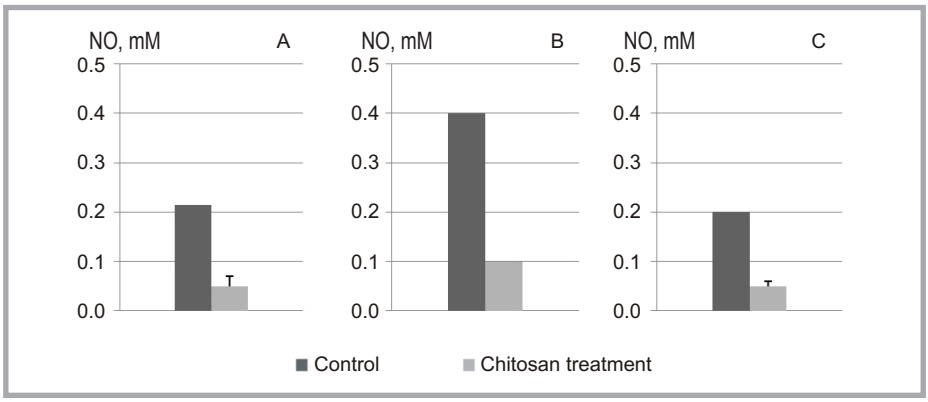

Figure 1. *Nitric oxide production (mg per ml of medium) in culture of peritoneal macrophages isolated from mice fed with chitosan; the level of NO: (A) spontaneous, (B) after LPS stimulation or (C) after chitosan re-stimulation.*

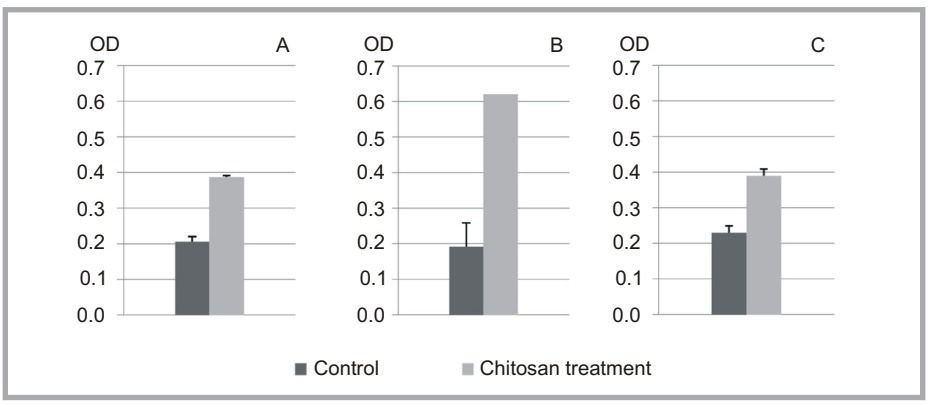

Figure 2. *Arginase activity (OD; optical density of urea visualised with isonitrosopropiophenone) in culture of peritoneal macrophages isolated from mice fed with chitosan; the level of urea: (A) spontaneous (B) after LPS stimulation or (C) chitosan re-stimulation.*

IL-4 and TGFβ in the gut. Also they observed that antigen is taken up mainly by chitosan presenting cells.

Activity of cells isolated from BALB/c mice which were treated alimentary with chitosan was evaluated. *In vitro* cell cultures were stimulated with LPS, a bacterial endotoxin, or re-stimulated with chitosan. After prolonged *in vivo* exposure to chitosan, peritoneal macrophages produced in culture less nitric oxide when compared to control, untreated animals (***Figure 1.A***). Those cells were also less responsive to LPS *in vitro* stimulation (***Figure 1.B***). At the same time, activity of arginase isolated from these cells was increased (***Figure 2.A***). Arginase activity increased even more after LPS stimulation (***Figure 2.B***), what suggests that activation status of these cells is Th2-type skewed. However, cells from

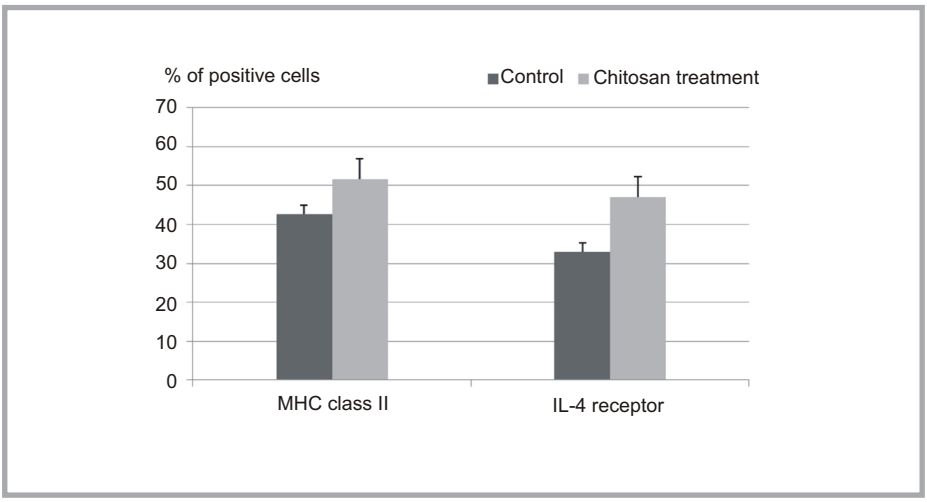

Figure 3. *Chitosan feeding increased the percentages of MHC class II and IL-4 receptor positive cells isolated from peritoneal cavity.*

both groups after 48 hours of *in vitro* re-stimulation with chitosan did not respond to treatment and NO production nor arginase activity was not affected (***Figures 1.C*** and ***2.C***).

In flow cytometry analysis elevated percentage of MHC class II positive cells was observed after alimentary chitosan treatment (***Figures 3***). It suggests that antigen uptake might be increased, yet it has to be determined if those cells express co-stimulatory molecules and can effectively activate effector cells. Some suggest [9] that fully potent immunostimulant activity of chitosan can observed only with simultaneous antigen exposition.

Also elevated percentage of IL-4R positive cells was observed after chitosan feeding (***Figures 3***). Increased expression of this receptor may imply that cells activated by chitosan can be susceptible to IL-4 activation and later may display alternative phenotype that is related to Th2 type responses.

Chitosan feeding effected in stimulation of peritoneal cells of mice. Reduced nitric oxide production with simultaneous elevated activity of arginase was observed after chitosan administration in drinking water only. As MHC class II protein level was elevated it suggests that chitosan is potent to alter antigen uptake and presentation as described before [9]. Further studies need to be done to verify if cells stimulated by chitosan are full active and can activate effector cells

4. Conclusions

We demonstrated that prolonged chitosan alimentary administration stimulates peritoneal innate cells of mice and it tends to polarise the response towards Th2 phenotype.

5. References

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