DIFFERENTIAL EFFECTS OF LOW AND HIGH MOLECULAR WEIGHT CHITOSAN ADMINISTERED INTRAPERITONEALLY TO MICE INFECTED WITH *HELIGMOSOMOIDES POLYGYRUS*

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Abstract
Aim of the study was to compare the effect of different molecular weight chitosan on activity of peritoneal cells of mice during immunosupression caused by adult stages of Heligmosomoides polygyrus. We observed that intraperitoneal injections of chitosan induce cell infiltration, but the activity of recruited cells differed depending on the type of polysaccharide used. Low molecular weight chitosan activated cells with inflammatory characteristics, while high molecular weight polysaccharide reduced cell responsiveness to stimulation. Although IgA titers in the peritoneal fluid were elevated, chitosan treatments had no effect on the level of infection.

Key words: *chitosan, parasite, immune response.*

1. Introduction

Multiple chitosan formulations have been proposed for application as vaccine adjuvant and drug carrier [**1, 2**]. These include nanoparticles [**3**], gels [**4**] and capsules [**5**]. They display several beneficial features due to chitosans mucoadhesivity, viscosity and ability to bind other molecules. It can release drugs in a controlled manner, protect transported molecule and, by loosening cell connections, facilitate absorption [**6**]. Known for biocompatibility, chitosan is generally regarded as a safe and well tolerated material; it was proven to have low oral toxicity [**7**] and is safe for topical use [**8**]. Yet, mixed information is available on its effect on immune functions when delivered to the host [**9**].

Multiple experiments show that chitosan administered with vaccine antigen enhances protection provided by antibodies [**10**]. At the same time, chitosan nanogels were shown to provide increased survival rate in test vaccines against neosporosis in murine model, but did not alter antibody titers [**11**]. Authors suggest that this effects was caused by the fact that chitosan influences innate immunity only. This can be supported by Koppulu and Zaharoff [**12**] who showed that chitosan enhances antigen uptake by phagocytic cells but itself does not alter marker expression on these cells. Chitosan was also proposed as a delivery agent in gene therapy. The polysaccharide was used both for transfection and gene silencing and showed no direct effect on cells and acted as a carrier only [**13**].

These variant conclusions may be due to the differences in methodology, i. e. chitosan preparation and characteristics. Different administration routes (subcutaneous, transmucosal or intraperitoneal) also provide various outcomes [**14**]. Therefore, more detailed studies need to be performed to establish biological properties and application potential of chitosan and its derivatives.

Aim of the study was to compare immunostimulant activity of chitosan of different molecular weight administered as intraperitoneal injections to mice infected with parasitic nematode *Heligmosomoides polygyrus*. This infection is a laboratory model to study mechanisms of immunosuppression caused by parasites that has both local and systemic consequences and may limit the efficacy of various treatment protocols, like vaccination [**14**].

2. Material and methods

2.1 Chitosan

Low molecular weight (LMW, 50 - 190 kDa, Degree of deacetylation; DD = 75 - 85%) and high molecular weight (HMW, 190 - 375 kDa, DD > 75%) chitosans were purchased from Sigma, Germany. Chitosans were prepared as 2% stock solutions in 0.75% adipic acid (Stanlab, Poland) and autoclaved.

2.2 Animals

Female C57Bl6 mice at the age of 8 weeks were orally infected with 180 L3 larvae of *Heligmosomoides polygyrus*. During the chronic phase of infection, 6 weeks post infection, animals were intraperitoneally injected with 500 μg of either chitosan solution for 10 days.

Animals were kept in a light:dark 12 h : 12 h cycle and were allowed *ad libitum* access to water and commercial pellet food. Just after treatment mice were euthanized. Each group consisted of at least 3 individuals. The experiment was conducted in accordance with the guidelines of the Local Ethical Committee.

2.3 Cell isolation and culture

Peritoneal cavity was washed with 5 mL of warm RPMI-1640 (Gibco, UK) supplemented with L-glutamine (2mM, Gibco, UK) and antibiotics (penicillin and streptomycin; 100 μg/ml, Gibco, UK) and the lavage was collected on ice. Cells were centrifuged at 1200 r.p.m. 4 °C for 7 min and washed with medium. Cells were counted with trypan blue exclusion, brought to concentration of $2{,}5\times10^6$/mL and seeded onto 96well plates, 200 μL per well. Cells were allowed to adhere to the surface for 2 h in 37 °C, 5% CO_2 and the unbound cells were washed away with warm medium. Remaining cells were activated with 200 μL of bacterial lipopolisaccharide (LPS, 2 μg/mL, Sigma, Germany) or left untreated in medium alone.

2.4 Measurement of nitrite and urea release

After 48 h of culture, cell supernatants were tested for nitric oxide production measured as presence of stable nitrate; 100 μL of culture medium was transferred to 100 μL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine, 3% phosphoric acid, all from Chempur, Poland) on a 96-well plate. After 10 min incubation, absorbance (OD; optical density) was measured at $\lambda = 550$ nm in a spectrophotomeater (μQuant, BioTek, USA) and concentration was calculated in relation to a standard curve of $NaNO^-_2$. Cells remaining on the plate were washed with warm PBS and used to measure arginase activity. Enzyme was released from cytosol by lysis in 0.1% Triton X for 30 min on ice. Then arginase was activated in 56 °C in the presence of 10 mM $MnCl_2$ for 15 min. After substrate addition, 0.25 M L-arginine, lysates were incubated for 1 h in 37 °C and then the reaction was stopped by 20% H_2SO_4/60% H_3PO_4/20% H_2O mixture. To visualise produced urea, 9% α-nitrosopropio-phenon in 99% ethanol was added and the reagents were heated to 95 °C for 1 h in the dark. (all reagents from Sigma, Germany). Absorbance of the product was measured in 100 μl of reaction solution at $\lambda = 540$ nm in spectrophotometer.

2.5 Measurement of antibodies

Level of specific immunoglobulin A (IgA) in peritoneal fluid was measured by enzyme-linked immunosorbent assay (ELISA). 96-well plate was coated with *H. polygyrus* adult somatic antigen and then blocked with 3% bovine serum albumin (BSA) in phosphatate buffered saline (PBS). Peritoneal lavages were incubated on plate for 2 h in room temperature and antibodies of IgA class were detected by addition of secondary antibody; murine IgA specific, horseradish peroxydase (HRP) labelled (Sigma, Germany). Reaction was visualised by addition of TMB/ H_2O_2 and stopped after 30 min with 1 N H_2SO_4. Absorbance was measured at $\lambda = 450$ nm in a spectrophotometer (μQuant, BioTek, USA).

2.6 Statistical analysis:

Results were considered significant when P value < 0.05 in student T test.

3. Results and discussion

Peritoneal cavity contains different cell populations with the majority of innate cells like monocytes, eosinophils and mast cells but also B lymphocytes [**15**]. It is a convenient surrounding to study the interface of innate and adaptive response to treatment. In our study, injection with low and high molecular weight chitosan caused marked cell infiltration to the peritoneum; more cells were present in the lavages (***Figure 1***). This effect was stronger when LMW chitosan was injected. In our preliminary studies, we observed no effect of adipic acid on cell activity, therefore here this control group is not included.

Adherent cells, after exposure to chitosan of various molecular weights, showed different activity in *in vitro* culture (***Figure 2.A*** and ***2.B***). LMW chitosan increased NO production with simultaneous diminished arginase activity what may suggest inflammatory response with reduced healing processes. HMW chitosan was not able to activate cells to produce NO; contrary it reduced the ability of cells to respond to LPS stimulation. Also arginase activity was strongly inhibited. These results may imply that LMW chitosan stimulate innate cells and induce inflammation while HMW diminishes cellular activity; cells tend to be areactive and do not respond to stimulation.

The effect of chitosan treatment could be seen also on the level of adaptive response as the IgA antibody titers in the peritoneal fluid were elevated after both types of chitosan were injected (***Figure 3***). Specific IgA response to *H. polygyrus* characterises mice with faster response to the infection [**16**]. It was shown that chitosan derivatives induce a high OVA-specific IgA in BALB/c mice [**17**]. In our studies, both humoral and cell-mediated immune responses induced by chitosan [**18**], are reflected in altered NO production and higher level of IgA.

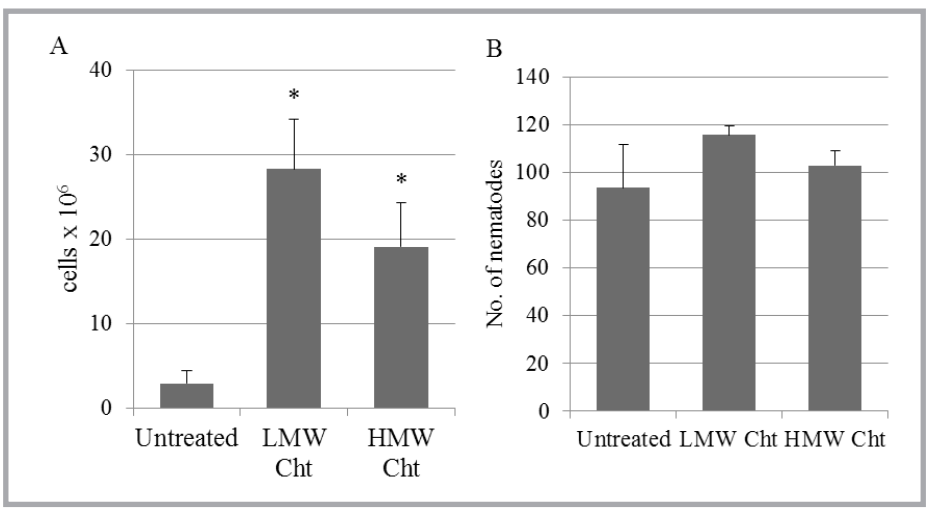

Figure 1. *Effect of low and high molecular weight chitosan on the number of (A) cells and (B) nematodes. Asterisks indicate P value < 0,05 in student T test versus Untreated group.*

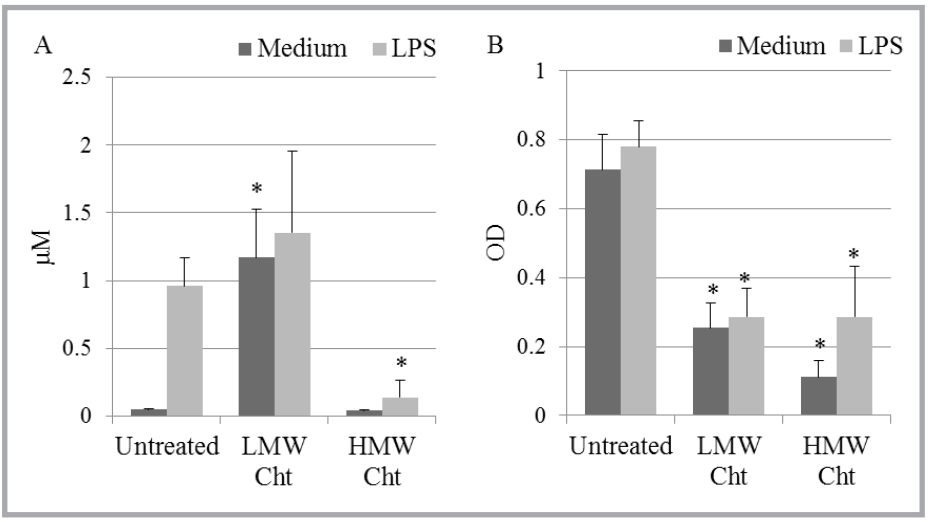

Figure 2. *Effect of low and high molecular weight chitosan on peritoneal cells activity after 48 h of in vitro culture; (A) nitric oxide production and (B) arginase activity. Asterisks indicate P value < 0.05 in student T test versus Untreated group.*

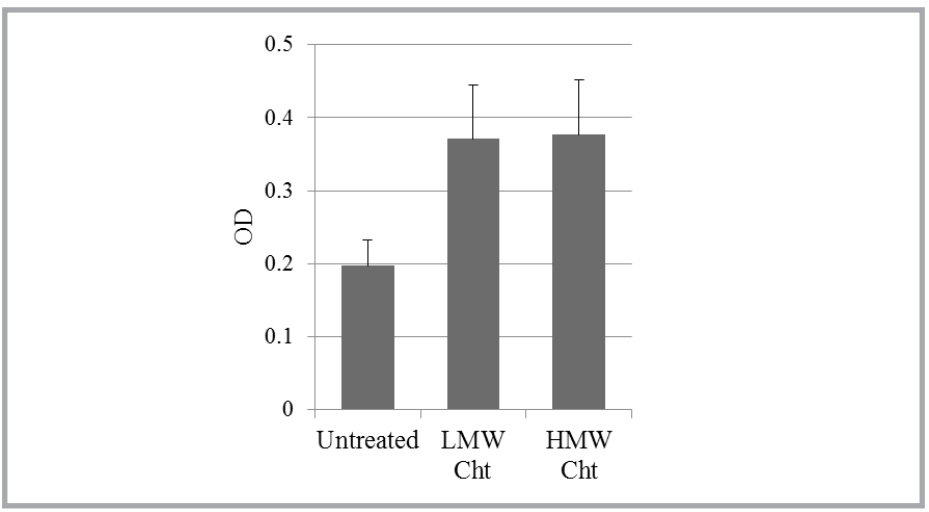

Figure 3. *Effect of low and high molecular weight chitosan on the level of IgA in peritoneal fluid.*

Despite the changes in the innate and adaptive immune mechanisms, no effect of chitosan treatment was seen on the number of nematodes (***Figure 1.B***). Level of infection remained unchanged, irrespective of the type of chitosan, and the polysaccharides did not induce raised parasite removal. The state of peritoneal cells activation measured *in vitro* is also not parallel to IgA production and number of worms. The IgA response is typical in immuneregulation [**19**] and IgA production primary induced by the infection is raised upon

chitosan treatment. In already adapted infection, chitosan administrated into peritoneal cavity is not potent to reverse the state of immunosuppression.

4. Conclusions

Activity of chitosan differs depending on its molecular weight. LMW polysaccharide induces inflammation-like mechanisms while HMW chitosan soothes cellular activity reducing cell response to stimulation. These features need to be taken under consideration when chitosan based pharmaceutical systems are developed. Choice of appropriate chitosan type may be crucial for the outcome of its biological effect and application.

5. References

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