

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

**Klaudia Brodaczewska, Maria Doligalska**

*Department of Parasitology,  
Faculty of Biology,  
University of Warsaw,  
Miecznikowa 1, 02-096 Warsaw, Poland  
E-mail: klaudiab@biol.uw.edu.pl*

**Abstract**

*Aim of the study was to compare the effect of different molecular weight chitosan on activity of peritoneal cells of mice during immunosuppression 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 chitosan's 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 effect 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  $2,5 \times 10^6$ /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<sub>2</sub> 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 spectrophotometer (µQuant, BioTek, USA) and concentration was calculated in relation to a standard curve of NaNO<sub>2</sub>. 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<sub>2</sub> 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<sub>2</sub>SO<sub>4</sub>/ 60% H<sub>3</sub>PO<sub>4</sub>/20% H<sub>2</sub>O mixture. To visualise produced urea, 9%  $\alpha$ -nitroso-propio-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 phosphate 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<sub>2</sub>O<sub>2</sub> and stopped after 30 min with 1 N H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at  $\lambda = 450$  nm in a spectrophotometer (µQuant, BioTek, USA).

### **2.6 Statistical analysis:**

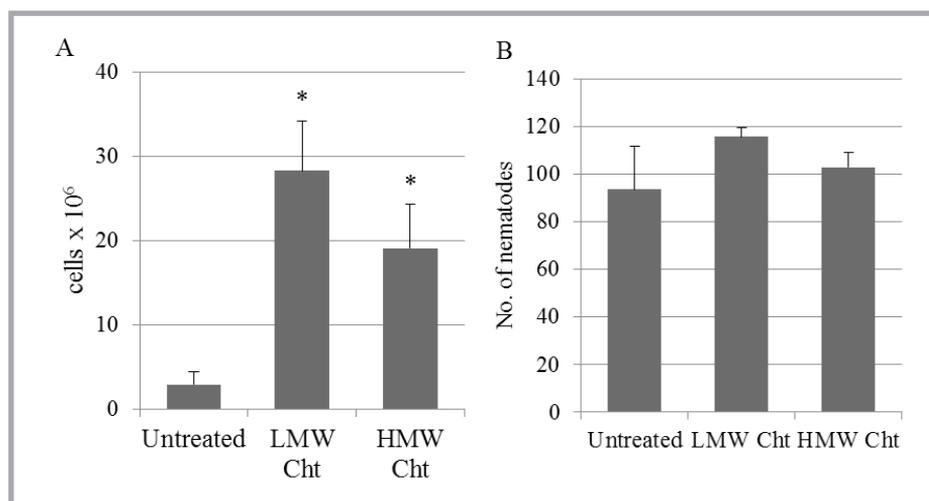
Results were considered significant when P value < 0.05 in student T test.

### 3. Results and discussion

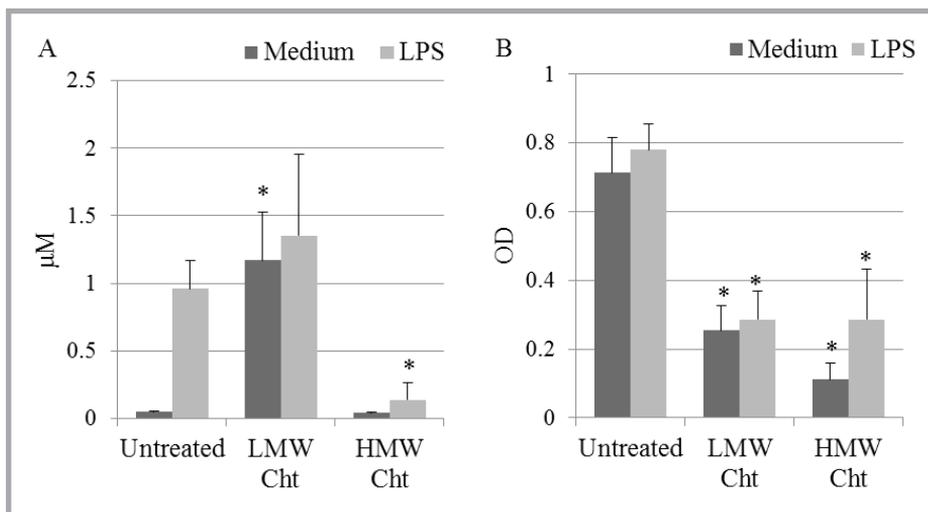
Peritoneal cavity contains different cell populations with the majority of innate cells like monocytes, eosinophils and mast cells but also B lymphocytes [15]. It is a convenient surrounding to study the interface of innate and adaptive response to treatment. In our study, injection with low and high 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.

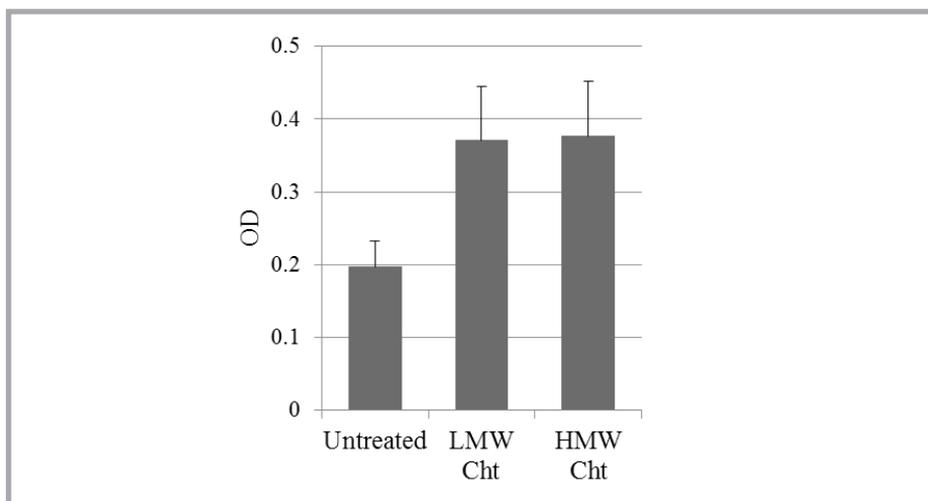
The effect of chitosan treatment could be seen also on the level of adaptive response as the IgA antibody titers in the peritoneal fluid were elevated after both types of chitosan were injected (**Figure 3**). Specific IgA response to *H. polygyrus* characterises mice with faster response to the infection [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 immunoregulation [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

1. Sinha VR, Singla AK, Wadhawan S, kaushik R, Kumria R, Bansal K, Dhawan S; (2004) Chitosan microspheres as a potential carrier for drugs. *Int J Pharm.* 274(1–2):1–33.
2. Senel S, McClure SJ; (2004) Potential applications of chitosan in veterinary medicine. *Advanced Drug Delivery Review* 56;1467–1480.
3. Wang JJ, Zeng ZW, Xiao RZ, Xie T, Zhou GL, Zhan XR, Wang SL; (2011) Recent advances of chitosan nanoparticles as drug carriers. *Int J Nanomedicine.* 6:765–774.
4. Neimert-Andersson T, Hällgren AC, Andersson M, Langebäck J, Zettergren L, Nilsen-Nygaard J, Draget KI, van Hage M, Lindberg A, Gafvelin G, Grönlund H; (2011) Improved immune responses in mice using the novel chitosan adjuvant ViscoGel, with a *Haemophilus influenzae* type b glycoconjugate vaccine. *Vaccine.* Nov 8;29(48):8965-73.
5. Tozaki H, Odoriba T, Okada N, Fujita T, Terabe A, Suzuki T, Okabe S, Muranishi S, Yamamoto A; (2002) Chitosan capsules for colon-specific drug delivery: enhanced localization of 5-aminosalicylic acid in the large intestine accelerates healing of TNBS-induced colitis in rats. *J Control Release.* 82(1):51-61.
6. Garcia-Fuentes M, Alonso MJ; (2012) Chitosan-based drug nanocarriers: where do we stand? *J Control Release.* Jul 20;161(2):496-504.
7. Qin C, Gao J, Wang L, Zeng L, Liu Y; (2006) Safety evaluation of short-term exposure to chito-oligomers from enzymic preparation. *Food Chem Toxicol* 44: 855–861.
8. Hirano S; (1996) Chitin biotechnology applications. *Biotechnol Annu Rev* 2: 237–258.
9. Oliveira MI, Santos SG, Oliveira MJ, Torres AL, Barbosa MA; (2012) Chitosan drives anti-inflammatory macrophage polarisation and pro-inflammatory dendritic cell stimulation. *European Cells and Materials* Vol. 24 2012 (pages 136-153).
10. Illum L, Jabbal-Gill I, Hinchcliffe M, Fisher AN, Davis SS; (2001) Chitosan as a novel nasal delivery system for vaccines. *Adv Drug Deliv Rev.* Sep 23;51(1-3):81-96.
11. Debache K, Kropf C, Schütz CA, Harwood LJ, Käuper P, Monney T, Rossi N, Laue C, McCullough KC, Hemphill A; (2011) Vaccination of mice with chitosan nanogel-associated recombinant NcPDI against challenge infection with *Neospora caninum* tachyzoites. *Parasite Immunol.* Feb;33(2):81-94.
12. Koppolu B, Zaharoff DA; (2013) The effect of antigen encapsulation in chitosan particles on uptake, activation and presentation by antigen presenting cells. *Biomaterials.* Mar;34(9):2359-69.
13. Huang Z, Dong L, Chen J, Gao F, Zhang Z, Chen J, Zhang J; (2012) Low-molecular weight chitosan/vascular endothelial growth factor short hairpin RNA for the treatment of hepatocellular carcinoma. *Life Sci.* Dec 10; 91(23-24): 1207-15.
14. Maizels RM, Hewitson JP, Murray J, Harcus YM, Dayer B, Filbey KJ, Grainger JR, McSorley HJ, Reynolds LA, Smith KA; (2012) Immune modulation and modulators in *Heligmosomoides polygyrus* infection. *Exp Parasitol.* Sep;132(1):76-89.

15. Ghosn EE, Cassado AA, Govoni GR, Fukuhara T, Yang Y, Monack DM, Bortoluci KR, Almeida SR, Herzenberg LA, Herzenberg LA; (2010) Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc Natl Acad Sci U S A*. Feb 9; 107(6): 2568-73.
16. Ben-Smith A, Wahid FN, Lammas DA, Behnke JM; (1999) The relationship between circulating and intestinal *Heligmosomoides polygyrus*-specific IgG1 and IgA and resistance to primary infection. *Parasite Immunol*. 21 (8): 383-395.
17. Kobayashi T, Fukushima K, Sannan T, Saito N, Takiguchi Y, Sato Y, Hasegawa H, Ishikawa K; (2013). Evaluation of the Effectiveness and Safety of Chitosan Derivatives as Adjuvants for Intranasal Vaccines. *Viral Immunol*. 26(2): 133-42.
18. Zaharoff DA, Rogers CJ, Hance KW, Schlom J, Greiner JW; (2007) Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. *Vaccine*. 25(11): 2085–2094.
19. Weiner HL; (2001) Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. *Microbes and Infection* 3(11): 947–954.

