

Modulatory effects of chitosan adipate on the T and B lymphocyte subsets in mice

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This study examined the subsets of T lymphocytes in the thymus, spleen and mesenteric lymph nodes as well as the subsets of B lymphocytes in the spleen and mesenteric lymph nodes in mice administered chitosan adipate (20 mg/kg) intraperitoneally once or four times at 24 h intervals. The results showed that chitosan adipate decreased the percentage of immature CD4⁺CD8⁺ thymic T cells and increased the percentage of mature CD4⁺ and CD8⁺ thymocytes. The most significant stimulating effect was observed after four injections. A single exposure to chitosan adipate increased the percentage of CD4⁺ mesenteric lymph node cells, but four injections of the drug increased the percentage of CD4⁺ and CD8⁺ mesenteric lymph node cells. Chitosan adipate had no effect on the subset of splenic T cells. In contrast, chitosan adipate administered either once or four times increased the percentage of CD19⁺ splenocytes but had no effect on the percentage of CD19⁺ mesenteric lymph node cells. Overall, chitosan adipate induces the maturation and differentiation of thymocytes, and regulates the number of B splenic cells and lymph node T cells irrespective of the number of doses.

Key words: B and T lymphocyte subsets, chitosan, mice

Introduction

A reduction in the number and function of immune cells can lead to a secondary immunological deficiency, which manifests as recurrent infections, autoaggression and cancer diseases. In the last three decades, a wide variety of substances, both natural (calf thymus extract, lysozyme dimer) and synthetic (levamisole, isoprinosine), have been introduced in order to modify the acquired defects of the immune cell activity.

Recently, it was reported that chitin derivatives have immunomodulatory action [5,13]. Chitosan (copolymer of β -91-4)-2-amino-2-deoxy-D-glucopyranose and β -(1-4)-2-acetamide-2-deoxy-D-glucopyranose) is an N-deacetylated product of chitin that is produced by chemical or enzymatic deacetylation resulting mainly in the removal of some or all of the acetyl groups from the acetylamino groups of chitin [1].

In vivo investigations have shown that chitin derivatives are most effective as an immune adjuvant [15,18,20]. van der Lubben *et al.* [18] reported that chitosan microparticles can enhance both the systemic and local immune responses against diphtheria toxoid (DT) after being administered orally and nasally in mice. The trials conducted on SRBC-immunized mice confirmed the potentiating effect of a water insoluble chitosan suspension on the humoral response in CBA mice [8]. This suggests that chitosan injected into mice causes an increase in the number of splenocytes producing haemolytic anti-SRBC antibodies (PFC) and the serum haemagglutinin titre. It was reported that chitin derivatives such as 70% deacetylated chitin (DAC-70) could activate peritoneal macrophages and natural killer (NK) cells, enhance the production of antibodies and delayed-type hypersensitivity in guinea pigs, and enhance the cell-mediated cytotoxicity in mice [12]. However there is little information on the influence of chitin derivatives on the T and B lymphocyte subpopulations. Therefore this study examined the effect of chitosan adipate on the surface marker expression of thymus, spleen and lymph node cells with respect to the treatment schedule.

Materials and Methods

Animals

The male and female Balb/c mice used in this study (weighing 18-20 g, 8-10 weeks of age) were obtained from a breeding laboratory at the Medical University, Wrocław, Poland.

The animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals (USA).

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Drug and treatment

Chitosan adipate at a dose of 20 mg/kg was administered intraperitoneally either once or four times at 24 h intervals. The process for synthesizing the chitosan adipate formed a part of project granted by the Polish Committee for Scientific Research (No 5PO6K04118) [1]. The volume of each chitosan adipate dose was 0.2 ml/mouse. The control mice received 0.2 ml of phosphate buffered saline (PBS) per mouse instead of chitosan adipate. Each experimental group contained eight mice.

Assay of thymocyte, splenocyte and lymphocyte of mesenteric lymph node subpopulations

The mice were anaesthetized with halothane 24 h after the final chitosan adipate injection. The thymus, spleen and mesenteric lymph nodes were removed and placed in disposable Petri dishes containing a sterile, ice-cold PBS. The suspended cells were released from the lymphatic organs by passing them through a nylon mesh and centrifuging them on a layer of Ficoll 400 (Pharmacia, Sweden)/Uropolinum 75% (diatrizoate sodium and meglumine diatrizoate; Polpharma, Poland) at a 1 : 3 ratio and a density of 1.071. After centrifugation at 4°C, the cells were collected from the interphase and washed twice with PBS supplemented with 1% bovine serum albumin (BSA; Sigma, USA) at 4°C. After the second wash, the cells were suspended in PBS with 1% BSA at 1×10^7 cells/ml. The viability of each cell suspension was 90-94% according to a trypan blue dye exclusion assay. The cells were resuspended in 100 µl PBS solution containing 1% BSA. The thymocytes, splenocytes and lymphocytes of the mesenteric lymph nodes were stained with the FITC-labeled antibody to the mouse CD4⁺ clone: YTS 177.9 (BioSource, Belgium) and the PE-labelled antibody to the mouse CD8⁺ clone: KT 15 (BioSource, Belgium) at the dilution recommended by the manufacturer. The splenocytes and lymphocytes of the mesenteric lymph nodes were also stained with the FITC-labelled antibody to the mouse CD3⁺ clone: KT3 (BioSource, Belgium) and the PE-labelled antibody to the mouse CD19⁺ clone: 6D5 (BioSource, Belgium) at a dilution recommended by the producer.

The cells were incubated at 4°C for 30 min. and washed 3 times with an ice-cold PBS buffer. The fluorescence was analysed using a flow cytometer (FACS Calibur; Becton Dickinson, Germany). The distribution of the thymocyte, splenocyte and lymphocyte markers was analysed using the Cell Quest program.

Statistical analysis

The data was analysed using a Student *t*-test. The differences were considered significant at *p* value <0.05.

Results

The trials conducted on mice confirmed the modulating effect of chitosan adipate on T and B-cell subpopulations originating from the lymphatic tissue. A relationship was observed between the stimulating effect induced by chitosan adipate and the number of doses applied. The strongest stimulating effect was noted after four injections of chitosan adipate (20 mg/kg) at 24 h intervals. The changes observed in the thymic cell subpopulations highlight the influence of chitosan adipate on thymocyte maturation and differentiation. The administration of chitosan adipate decreased the percentage of immature CD4⁺CD8⁺ thymic cells (double-positive cells) with corresponding increases in the percentage of single-positive CD4⁺ and CD8⁺ thymocytes. The stimulating effect depends on the number of chitosan adipate doses applied. The strongest effect was noted after four injections of chitosan adipate at a dose of 20 mg/kg (Table 1).

Some changes in the percentage of mesenteric lymph node T cells were also found. The administration of chitosan adipate once at 20 mg/kg led to an increase in the percentage of T CD4⁺ mesenteric lymph node cells but did not affect the percentage of T CD3⁺ and CD8⁺ lymphocytes from the mesenteric lymph nodes. Exposure to four chitosan adipate doses increased the percentage of T CD4⁺ and CD8⁺ mesenteric lymph node cells. On the other hand, there were no significant changes in the percentage of T splenocyte subpopulations.

Irrespective of the number of chitosan adipate doses applied, there was an increase in the percentage of B CD19⁺ mesenteric lymph node cells but there was no effect on the percentage of B CD19⁺ splenocytes.

Discussion

It is likely that the modulating effect of chitosan adipate on the subpopulations of T lymphocytes in the thymus and peripheral lymphatic organs (lymph nodes) is related to synthesis and release cytokines, particularly interleukin-1 (IL-1) and interleukin-2 (IL-2). Nishimura *et al.* [11] reported that deacetylated chitin (DAC-70) induces the synthesis and release of cytokines such as IL-1, colony-stimulating factor (CSF) and interferons (IFN) in mice. IL-1 plays a supporting role in the activation of T cells, partly by increasing the expression of the IL-2 receptors on thymocytes [6,19] and enhancing the proliferation of PHA-stimulated thymocytes [3,9]. It is known that the maturation of T lymphocytes not only occurs in the thymus but in other lymphatic organs as well.

The results also show that chitosan adipate administered to mice increases the percentage of B (CD19⁺) cells in the

Table 1. Percentage of thymocytes, splenocytes and lymphocytes from the mesenteric lymph node subpopulations in the mice treated with chitosan (n = 8, mean ± SD)

Index	Day	Control	Chitosan single administration	Chitosan four times administration
Thymocytes				
CD4 ⁺ CD8 ⁺	1	65.1 ± 4.8	58.5 ± 5.0*	53.6 ± 5.1*
CD4 ⁻ CD8 ⁻	1	17.1 ± 4.7	17.9 ± 3.3	17.6 ± 2.7
CD4 ⁺	1	11.5 ± 1.7	15.9 ± 2.5*	19.5 ± 1.5**
CD8 ⁺	1	6.3 ± 0.8	7.7 ± 1.3*	9.3 ± 1.9*
Splenocytes				
CD3 ⁺	1	25.7 ± 5.4	28.2 ± 1.4	28.4 ± 1.6
CD4 ⁺	1	19.0 ± 4.0	20.5 ± 1.3	19.9 ± 1.5
CD8 ⁺	1	5.6 ± 1.4	6.1 ± 1.9	6.9 ± 1.2
CD19 ⁺	1	44.2 ± 8.1	54.8 ± 2.1*	52.3 ± 4.3*
Mesenteric lymph node cells				
CD3 ⁺	1	37.6 ± 5.5	36.2 ± 7.9	38.1 ± 2.8
CD4 ⁺	1	17.8 ± 3.3	23.5 ± 3.7*	25.6 ± 1.4*
CD8 ⁺	1	7.4 ± 4.3	6.2 ± 0.8	13.5 ± 2.2*
CD19 ⁺	1	37.8 ± 4.6	38.2 ± 8.1	38.6 ± 5.6

Note: Chitosan (20 mg/kg) treated once or four times at 24 h intervals.

* $p < 0.05$ compared with the control group.

** $p < 0.05$ compared with the group treated with a single dose

spleen but does not alter the percentage of B lymphocytes in the lymph nodes. It is likely that the stimulating effect of chitosan adipate on the number of B splenocytes is due to the activation of T lymphocytes and macrophages through the cytokine cascade (IL-1, CSF and IFN- γ) that is enhanced by this agent. These factors activate the functions of polymorphonuclear cells (PMNs) and macrophages [7,10, 14,16]. IL-1 can induce the maturation of pre-B-cells [4]. This cytokine acts synergistically with various B cell growth factors to augment proliferation, increase the level of immunoglobulin production, and induce or augment T cells for the production of IL-2, IL-4, IL-5 and IL-6, all of which have immunomodulatory effects on B cells [2]. Interferons have a significant effect in B-cell growth and differentiation. IFN- γ acts as a regulatory agent in determining the responses to the immunoglobulin isotype. This agent selectively enhances the production of IgG_{2a} while simultaneously suppressing IgG₁, IgG_{2b} and IgG₃ synthesis [17]. Iida *et al.* [5] reported that the intranasal administration of chitin derivatives (DAC-70) enhanced the production of IFN in the lung one day after a *Sendai virus* infection. In addition, DAC-70 exhibited protective activity against *Sendai virus* and an *E. coli* infection in mice. However, they reported that the protective effect of DAC-70 depended on the treatment schedule and the route of drug administration.

In conclusion, chitosan adipate (20 mg/kg) administered either as a single dose or four times induces the maturation and differentiation of thymocytes and regulates the number of specific cluster differentiation antigens on the surface of B splenocytes and lymph node T cells.

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