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Influence of physico-chemical properties of chitin and chitosan on complement activation

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Abstract

To recognize the complement activation by chitin and chitosan, various physico-chemical aspects were studied. Complement activation was determined by change of plasma C3 concentration using single radial immunodiffusion method. Results were as follows: in samples with homogeneous acetylation (oDAC), C3 concentration was decreased with the increase in the degree of acetylation of oDAC. In oDACs 50 and 42 (water soluble materials), however, C3 was not decreased. In samples with heterogeneous acetylation or deacetylation samples (eDAC), all samples showed C3 activation even in eDAC 53 (solid material) and have almost same activity on complement activation. C3 activation was not seen in low molecular weight materials including D-glucosamine. The important factors inducing complement activation by chitosan-based mucopolysaccharide should be solids. The samples with heterogeneous acetylation (eDACs) had a stable ability on complement activation than with homogeneous acetylation (oDACs). © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Chitin; Chitosan; Complement activation; Dog; Physico-chemical properties

1. Introduction

Various biological activations induced by chitin or chitosan were investigated. Subcutaneous administration of chitosan at the rate of 1 mg/kg (Minami et al., 1997a) or intravenous administration of chitin at the rate of 1 mg/kg (Minami et al., 1997b) induced temporary chemiluminescence activity of canine polymorphonuclear cells during approximately 7 days. Further, chitosan overdosing (subcutaneous administration of 200 mg/kg) brought about severe pneumonia in dogs (Minami, Oh-oka, Okamoto, Miyatake, Matsuhashi & Shigemasa, 1996). This reaction had a close resemblance to acute respiratory distress syndrome (ARDS) (Murray, 1977) in human beings and to the model of ARDS in mice brought about by venous injection of cobra venom (Mulligan, Paulson, De Frees, Zheng, Lowe & Ward, 1993). The mechanism of biological activation on wound healing acceleration and complement activation by chitin and chitosan was well demonstrated in the previous in vitro study (Minami, Suzuki, Okamoto, Fujinaga & Shigemasa, 1998). On single radial immunodiffusion tests for C3, C4, and C5 after incubation with chitosan, C3 and C5 serum concentrations were decreased, but not that of C4. These results demonstrated that complement activation occurs via an alternative pathway, with an effect similar to that of zymosan, but its detailed mechanisms are unknown.

In the present paper, the possible mechanisms of complement activation by chitin and chitosan were studied with respect to its physico-chemical characteristics such as degree of deacetylation and water solubility.

2. Materials

2.1. Animals

Three healthy male beagles (1–2 years old, weighing 10–12 kg) all showing normal values upon routine blood and physical examination, were used for the collection of normal plasma.

2.2. Experimental agents

Preparations of partially acetylated chitosan in homogeneous state: Chitosan powder was prepared from commercial chitosan flakes (Koyo Chemical Co. Ltd, Tokyo, Japan) with degree of deacetylation (DDA) 82%

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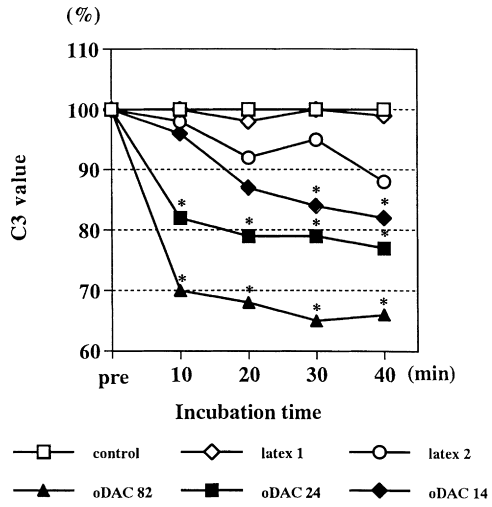

Fig. 1. Effect of solid materials (latex and oDAC) on C3 activation. Control: physiological saline; latex 1: latex suspension (particle size: 1 μm); latex 2: latex suspension (particle size: 2.8 μm); oDAC: homogeneous partially acetylated (14, 24 and 82%) chitosan particle suspension. The concentration of each agent is 10 mg/ml. 100 μl of each agent were mixed with 100 μl of canine serum and then incubated at 37°C. Individual data were converted to % relative to the initial complement level (pre-incubation). Statistical analysis was performed between each initial level and after incubation level (* : $p < 0.05$).

and molecular weight approximately 80,000, by pulverizing into average of particle 5.8 μm using ball mill (Ube Industries Co. Ltd, CF-400, Ube). The powder was completely deacetylated (DDA 100) with the method described by Mima, Miya, Iwamoto and Yoshikawa (1983). Homogeneous partial acetylation was performed with the method described by Hirano, Ohe and Ono (1976). The DDAs of the final products were 82, 50, 42, 24, and 14, respectively.

Preparations of partially acetylated chitosan in heterogeneous state: Heterogeneous acetylation or deacetylation of the powder was performed by the method described by Miya, Iwamoto, Ohta and Mima (1985). The DDAs of the final products were 100, 53, 40, 33, and 14, respectively. In this paper, the homogeneous and the heterogeneous products are expressed by oDAC for homogeneously prepared one or eDAC for heterogeneously prepared one.

DDA was evaluated by the IR method (Shigemasa, Matsuura, Sashiwa & Saimoto, 1996) and/or by the colloidal titration method (Kina, Tamura & Ishibashi, 1974). Distributions of particle size of all samples were measured by laser diffraction particle size analyzer (Shimazu Co. Ltd, SALD-2000 A, Tokyo, Japan). All samples were sterilized by ethylene oxide gas and then suspended or dissolved (oDACs 50 and 42) in physiological saline.

Chitosan oligosaccharides: Two types of soluble chitosan oligosaccharides; oligo 1: with MW 3000–30,000 (Wako Pure Chemicals Ltd, Osaka, Japan) and oligo 2: with MW 216–1204 (Yaizu Suisankagaku Industries Co. Ltd, Shizuoka, Japan) were dissolved in physiological saline at the concentration of 10 mg/ml.

D-*glucosamine*: D-glucosamine HCl (Yaizu Suisankagaku Industries Co. Ltd, Shizuoka, Japan), was dissolved by physiological saline in the concentration of 10 mg/ml.

Latex beads: Latex beads (particle mean diameters: 1.0 μm (latex 1) and 2.8 μm (latex 2)) were purchased from Polyscience Inc. (Polybead R 2.5% Solids-Latex, USA), and suspended in a concentration of 10 mg/ml by physiological saline, respectively.

Complement anti-serum: Anti-dog C3 goat serum was purchased from ICN Pharm., Inc. (USA) and dissolved in 2 ml of the distilled water.

3. Methods

3.1. Blood collection and examination

Blood samples were collected from the jugular vein of each dog by heparinized sterile syringe using 18-gauge needle and apportioned into cooled test tubes stored in an ice bath, followed by centrifugation at 4°C, 1200 *g* for 5 min. Then the collected plasma was separated into tubes and kept in a deep freeze at −80°C until use.

The stored frozen plasma was thawed in the water bath at 37°C and then divided into four test tubes (each 100 μl). Each tube was tested as followed: the first test tube was used for a measurement of the basic value representing the original C3 concentration in the plasma (plasma + 100 μl physiological saline without incubation); the second tube represented the positive control of chitosan (oDAC 82) activated plasma (plasma + 100 μl oDAC 82 suspension, with incubation at 37°C for 10, 20, 30, and 40 min, respectively); the third tube was used for the experimental agent (other oDACs, eDACs, D-glucosamine, chito-oligosaccharides, and latex beads, respectively) viz, the plasma for the test (plasma + 100 μl experimental agent, with incubation at 37°C for 10, 20, 30, and 40 min, respectively). The final tube represented the negative control i.e. the effect of incubation on the concentration of C3 (plasma + 100 μl physiological saline with incubation at 37°C for 10, 20, 30, and 40 min, respectively). Each experiment was done in triplicate.

3.2. Complement assay

All tubes were centrifuged at 4°C, 1200 *g*, for 5 min after treatment, and the supernatants were collected and used for estimation of C3 concentration by single radial immunodiffusion (SRID). The method of SRID is summarized as follows: a gel was made by 1.5 g of purified agar (Sigma Chemical Co., USA) and 30 mg of sodium azide (Nakarai Tesque Co., Tokyo, Japan) dissolved in 100 ml of phosphate buffer by microwave treatment for 2 min. The gel reheated in hot water for 30 min was then kept in the water bath at 50°C. Then 150–200 μl of anti-dog C3 serum was added to 6 ml of the gel. To make a gel plate, the gel was poured into a square glass box, and immediately cooled in the ice bath.

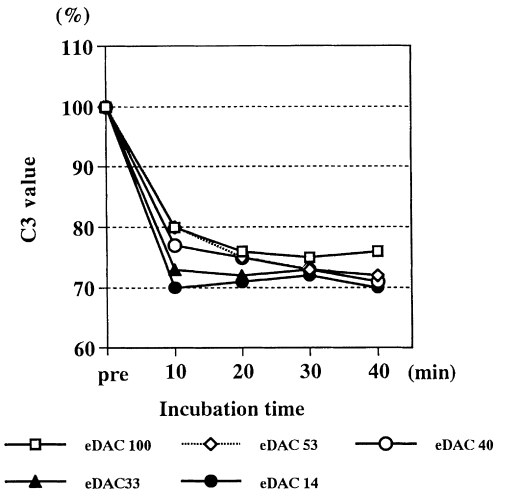

Fig. 2. Effect of solid materials (eDAC) on C3 activation. eDAC: heterogeneous partially acetylated (14, 33, 40, 53 and 100%) chitosan particle suspension. The concentration of each agent is 10 mg/ml. 100 μl of each agent were mixed with 100 μl of canine serum and then incubated at 37°C. Individual data were converted to % relative to the initial complement level (pre-incubation). In all samples, significant decrease was seen between each initial level and after incubation level.

The gel plate was removed from the flat glass plate, and sectioned by cutting with a sharp blade into pieces of 1×1 cm^2, after which a 1.5 mm diameter hole was made in the center of each plate. The gel was kept in a humidified box. Each plasma specimen was placed in the center hole and the plate was incubated at 4°C for 48 h. After incubation, the size of the precipitation ring was measured using a stereoscopic microscope. The calculation formula of the C3 value is as follows: ring size $= \pi(R_a^2 - R_b^2)$, where R_a is half of the ring diameter and R_b is half of the center hole diameter. Each ring size (C3 value) was converted into a percentage by each base value ring size (100%).

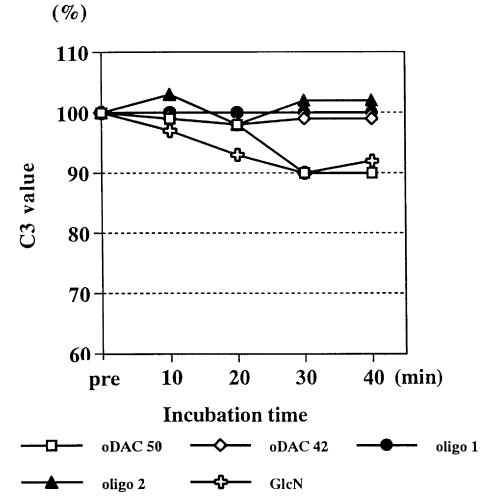

Fig. 3. Effect of water soluble materials on C3 activation. oDAC: homogeneous partially acetylated (42 and 50%) chitosan solution; oligo 1: chitosan oligosaccharides (MW 3000–30,000); oligo 2: chitosan oligosaccharides (MW 216–1204); GlcN: D-glucosamine. The concentration of each agent is 10 mg/ml. 100 μl of each agent were mixed with 100 μl of canine serum and then incubated at 37°C. Individual data were converted to % relative to the initial complement level (pre-incubation). In all samples, there was no significant difference between each initial level and after incubation level.

4. Results

4.1. Effect of solid materials on C3 activation

Two types of latex beads and all oDACs, eDACs except oDACs 42 and 50 were tested. There was no significant change in C3 values in latex 1 (1.0 μm dia.), and latex 2 (2.8 μm dia.) of groups except insignificant decrease of C3 value (88.4%) at 40 min (Fig. 1). Regarding the effects of oDAC (Fig. 1) on complement activation, C3 values were significantly decreased to 70 and 82% levels by oDACs 82 and 24, respectively, when compared with the negative control after 10 min of incubation. Value of oDAC 14 was gradually decreased, with significant decrease at 30 and 40 min of incubation.

C3 values were decreased to approximately 72–80% at 10 min by all eDAC samples with no correlation with the DDA, even by eDAC 53 and maintained at the same level until 40 min (Fig. 2).

4.2. Effect of water soluble material on C3 activation (Fig. 3)

oDACs 42, 50, chitosan oligosaccharides and D-glucosamine did not show any significant change on C3 value.

5. Discussion

We had reported that chitin and chitosan caused complementary activation via an alternative pathway (Minami et al., 1998), but physico-chemical characteristics of agents that include complementary activation were not clear at that time. Chitin is a natural acetylated chitosan, and chitosan also can be expressed as deacetylated chitin. Therefore, determining the effects of various degrees of partially acetylated or deacetylated (DAC) materials on complementary activation is one of the keys to identify the mechanism of complement activation with chitin and chitosan. We prepared 10 different acetylated or deacetylated materials which the same molecular weight. Half of them were prepared from completely deacetylated chitin (DAC 100) in homogeneous state by acetylation (oDACs 50, 42, 24, 14) technique, and the others were made from DAC 82 in heterogeneous state by deacetylation (eDAC 100) or acetylation (eDACs 53, 40, 33, 14) techniques. All heterogeneous materials showed complement activation and their abilities for complement activation were almost same. On the other hand, for homogeneous materials, the intensity of complement activation was decreased with the increase in degree of acetylation. Difference between heterogeneous and homogeneous materials on complement

activation is unknown, but it may be possible for random or block chemical reaction to occur. In homogeneous materials, acetylation would be more random than heterogeneous materials.

oDACs 42 and 50 did not activate C3. It is well known that oDACs with approximately a 50% degree of acetylation have water solubility (Kurita, Sannan & Iwakura, 1977), and oDACs 42 and 50 were also dissolved in physiological saline. Further, it was clear that D-glucosamine and its short chain materials, were water soluble and did not activate C3. Generally, the number of amino groups will correspond to the intensity of complement activation, but low molecular or water soluble muco-polysaccharides, even if they are DAC 100, would not demonstrate this effect. In preliminary experiments, water soluble high molecular chitin derivatives, such as sulphated, phosphated, or carboxymetylated chitins, did not demonstrate correspondingly high complement activation.

The amino group played an important role in C3 activation, and with the results of the latex beads, it is clear that physical stimulation alone cannot cause activation of C3.

Nevertheless, it was not clear how these materials activated C3. The initial activation of the alternative pathway is explained as follows: (1) C3b formation by protease (Pangburn & Muller-Eberhard, 1980); (2) C3i formation by hydrolysis of thioester residue in C3 (Pangburn & Muller-Eberhard, 1980; Pangburn, Schreiber & Muller-Eberhard, 1981); and (3) C3i formation by vibration (Law, 1983). Serial important chemical reactions are reported as C3b or C3i binding to hydroxyl or amino groups on cell membrane and their stabilization as membrane-combined C3b or C3i to continue complement activation cascade. These combined components act as the C3-transfer enzyme with factors B and D; this enzyme reacts with properdin. Properdin increases the life of the C3/C5-convertases (Law & Reid, 1995). For the above reason, C3b must be fixed for continuity of complement activation. To put it simple, chitosan and chitin play a very important role for fixation of these components because they have many amino and hydroxyl groups. Chitosan and chitin may act directly to change C3 to C3i, or indirectly to activate serum proteases, but the details of this mechanism are yet to be elucidated. Further investigation should be conducted.

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