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Chitin and chitosan activate complement via the alternative pathway

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Abstract

The effect of chitosan on the serum C3 concentration was investigated in dogs and mice after subcutaneous administration. Chitosan (10 mg/kg) induced an increase of the C3 level in dogs, but not in mice. To attain the same C3 level in mice as in dogs, the dose of chitosan had to be increased five-fold. Chitin and chitosan activated complement components C3 and C5, but not C4. The intensity of complement activation was greater with chitosan than with chitin. Chitin and chitosan both activated complement via the alternative pathway. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: Chitin; Chitosan; Alternative pathway

1. Introduction

We have already reported that subcutaneous administration of chitosan (200 mg/kg) induces severe hemorrhagic pneumonia in dogs (Minami et al., 1996), and that administration of 10 mg/kg induces systemic activation of the chemiluminescence response (CL) in canine peripheral polymorphonuclear cells (PMN) (Minami et al., 1998). The increased CL response was observed in PMN recovered from chitosan-treated dogs after incubation with normal serum and in the PMN of an untreated dog after incubation with serum recovered from chitosan-treated dogs. These findings showed that activation of both PMN and serum was induced by administration of chitosan to dogs. The effect of serum on PMN was decreased by heating at 56°C for 30 min, suggesting,that this effect was brought about by complement activation, especially the activation of thermolabile factors (Minami et al., 1993).

In vitro experiments using chitin and chitosan stimulate the CL response of PMN to normal serum showed that chitin and chitosan induced 30% and 50% of the zymosan-induced CL (100%), respectively. In the absence of serum or in the presence of heat-treated serum, chitin and chitosan did not induce a CL response of canine PMN (Minami et al., 1993). However, the supernatant of serum incubated with chitin or chitosan increased the CL response to zymosan of PMN recovered from untreated dogs and this effect was decreased by heat treatment (Minami et al., 1993). Serum treated with chitin or chitosan also increased the number of migrating PMN in an experiment using the Boyden chamber (Usami et al., 1997).

Chitin and chitosan were observed to accelerate wound healing and to promote the attainment of good skin healing without scarring (Chandy and Sharma, 1990; Okamoto et al., 1993). Histological findings suggest that these materials stimulate the migration of PMN and mononuclear cells, and accelerate the regeneration of vascular granulation tissue (Minami et al., 1993; Muzzarelli et al., 1988).

In the present study, we focused on the role of the complement in biological activation by chitin and chitosan.

2. Experimental

2.1. Animals

Six normal adult beagle dogs (1–3 years old and weighing 8–15 kg) and 39 *ddy* mice (6 weeks old) were used for assessment of systemic complement activation after subcutaneous administration of chitosan.

2.2. Normal human serum

To investigate the direct effects of chitin and chitosan,

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fresh normal human serum collected from three healthy male volunteers was used.

2.3. Agents

Chitin and chitosan suspensions: Commercial squid pen chitin purified from the Neon flying squid (*Ommastrephes bartrami*) (Nippon Suisan Co., Ltd., Tokyo, Japan) was used, which comprised β-chitin with 9% deacetylation and an average molecular weight > 100 000. Chitosan flake (Flonac C, Kyowa Tecnos Co., Ltd., Japan) was also used which was 82% deacetylated α-chitin, purified from crab shell and had an average molecular weight of 80 000, maximum 1.2% ash, and a maximum 5 ppm of heavy metals like Pb, Cd, and As. Both preparations were pulverized into 3 μm particles with a mill (Ube Industries, Ltd., Japan, CF-400). These fine powders were sterilized by ethylene oxide gas, and were suspended in physiological saline at a concentration of 30 mg/ml for dogs and 5 mg/ml for mice (the original suspension).

Zymosan suspension: after 0.1 g of zymosan (Sigma, USA) was suspended in 10 ml of physiological saline, the suspension was centrifuged at 2000 rpm for 10 min, and then diluted with HEPES plus Hank's balanced salt solution (HEPES–HBSS, phenol red free, Nihon-suisan Co. Ltd., Tokyo, Japan) to 10 ml after decantation.

Luminol solution: luminol was diluted to 2 mg/ml with HEPES–HBSS, and then 50 μl of triethylamine (Wako, Tokyo, Japan) was added to this solution. After ultrasonication for 45 min, this solution was passed through a 0.45 micron Millipore filter in order to remove insoluble Luminol particles.

Anti-complement serum: 1 mg of anti-dog C3 goat-serum (Bethyl Laboratories, Inc., USA), anti-mouse C3 goat serum (Bethyl), anti-human C3 goat serum (Bethyl), anti-human C4 goat serum (Bethyl), and anti-human C5 goat serum (Bethyl) was dissolved in 2 ml of physiological saline and stored at − 80°C.

Phosphate buffer: A total of 57.8 g of disodium hydrogen phosphate 12 hydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 5.92 g of disodium hydrogen phosphate dehydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were dissolved in an adequate volume of distilled water and then this solution was diluted to 1 l with distilled water.

Agarose gel: After 1.5 g of purified agarose powder (Nacalai Tesque, Inc., Kyoto, Japan) was dissolved in 100 ml of phosphate buffer, 30 mg of sodium azide (Nacalai Tesque, Inc., Kyoto, Japan) was added.

3. Methods

3.1. Effect of subcutaneous chitosan on complement

Six dogs were divided into two groups of three animals and were given 10 mg/kg of chitosan or no chitosan as control. Each dog in the chitosan group was administered the chitosan dose at two sites in the subcutaneous tissue of the neck using a syringe with a 20 gauge needle. The injection volume was adjusted to 1 ml/kg by dilution of the original suspension with physiological saline. In the control group, 1 ml/kg of physiological saline was injected subcutaneously in the same manner as in the chitosan group. The mice were divided into three groups, including a 10 mg/kg chitosan group ($n = 12$), a 50 mg/kg chitosan group ($n = 12$), and a control group ($n = 15$). In the chitosan groups, 350 μl of chitosan suspension was prepared in a syringe with a 24 gauge needle by dilution of the original suspension with physiological saline and was administered subcutaneously into the back. In the control group, 350 μl of physiological saline was administered in the same manner as in the chitosan groups.

3.2. Blood collection and examination

In the dogs, 5 ml of blood was collected from the jugular vein before chitosan or physiological saline administration (Pre), and at 3 days (day 3) and 6 days (day 6) after administration. The blood tests described below were performed with 1 ml of the collected blood, and another 1 ml was used for the CL assay. The red blood cell count (RBC), white blood cell count (WBC), each differential white blood cell count, and hemoglobin concentration (Hb) were measured in the routine manner. The serum concentration of complement component 3 (C3) was also measured, using serum separated from another 3 ml of blood by centrifugation at 3000 rpm for 30 min at 4°C. Blood was collected from three mice each by direct cardiac puncture under ether anesthesia before administration of physiological saline (Pre), and at 3 days (day 3) and 6 days (day 6) after administration. In the chitosan groups, blood was collected from five mice at 3 and 6 days after administration of chitosan. The aliquots of collected blood were pooled at − 80°C and half were used for same tests as described for the dogs. C3 was assayed after separation of serum by centrifugation at 3000 rpm for 30 min at 4°C using the remaining blood samples.

In human volunteers blood was collected from the cephalic vein in the Tottori University, Health Care Center, and was separated by centrifugation at 3000 rpm for 30 min at 4°C as soon as possible. Serum for complement assay was stored at − 80°C before use.

3.3. Chemiluminescence (CL) by blood

Measurement of CL according to Makimura and Sawaki (1992) was done with a Lumat LB-9501 (Berthold Co., Germany). In brief, blood was diluted five-fold with HBSS in a cuvette and was incubated at 37°C for 15 min, after which 20 μl of Luminol (2 mg/ml) was added to the cuvette. After incubation for 5 min more, measurement was

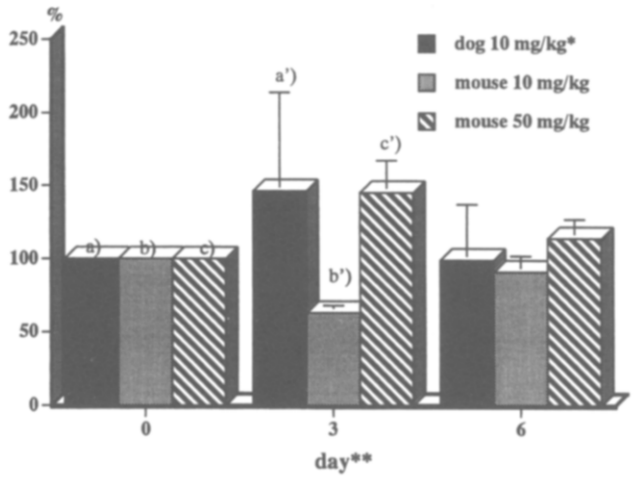

Fig. 1. Effect on the chemiluminescence index of peripheral polymorphonuclear cells after subcutaneous injection of chitosan. *, 10 mg/kg of chitosan was administered subcutaneously. **, Day 0 means before injection (Pre.) days 3 and 6 means days after injection. (a') and (c') are significantly increased compared with (a) and (c) ($p < 0.01$). (b') is significantly decreased compared with (b) ($p < 0.01$).

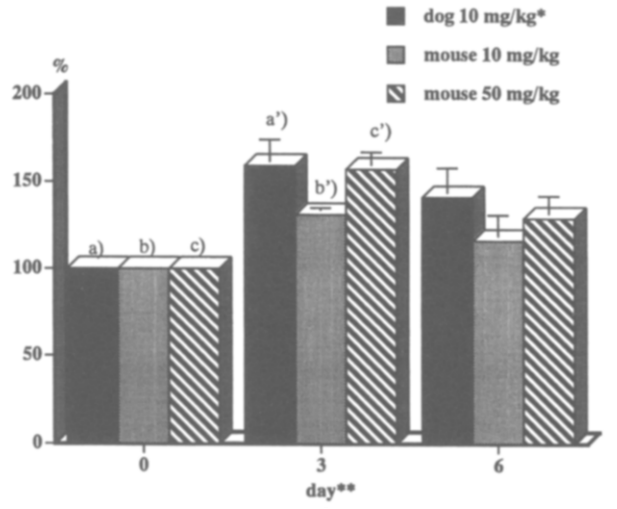

Fig. 2. Effect on C3 concentration in peripheral after subcutaneous injection of chitosan. *, 10 mg/kg of chitosan was administered subcutaneously. **, Day 0 means before injection (Pre.) days 3 and 6 means days after injection. (a') and (c') are significantly increased compared with (a) and (c) ($p < 0.01$). (b') is significantly decreased compared with (b) ($p < 0.01$).

started. The initial 1 min was used to obtain the baseline value and then 50 μl of zymosan (10 mg/ml) was added to the cuvette as a stimulator and the peak CL response over 15 min was measured. The intensity of CL per 1000 PMN (CL index) was calculated as follows:

CL index = {peak CL × (1 − baseline index)/G × V} × 1000, where the baseline index is the baseline value divided by the peak value, G is the number of PMN per microliter of blood, and V is the measured blood volume (100 μl).

3.4. Complement assay

The levels of C3, C4, and C5 were assayed by the single radial immunodiffusion method. The optimum dilution of each anti-serum was decided in a preliminary study.

Anti-dog C3 goat serum, anti-mouse C3 goat serum, anti-human C3 goat serum, antihuman C4 goat serum and anti-human C5 serum was diluted 20-, 40-, 40-, 80-, and 30-fold, respectively, in agarose gel warmed at 50°C, and then the gel was hardened in ice water. A central hole was created in the gel with a small corkscrew and 2.5 μl of test serum was placed in the hole, followed by incubation for 48 h at 4°C.

3.5. Treatment of human serum with chitin and chitosan

Fresh human serum (1 ml) was incubated with a chitosan (1 ml) or zymosan (1 ml) suspensions at a concentration of 0.1, 1, and 10 mg/ml at 37°C for 10, 20, 30 and 40 min. Each supernatant was recovered by centrifugation at 3000 rpm for 5 min under 4°C. Chitin suspension was also incubated with serum in the same manner at concentrations of 0.1 and 1 mg/ml. Control supernatant was prepared by incubation with physiological saline (1 ml) and serum (1 ml). All of these supernatants were subjected to analysis of complement.

4. Results

4.1. Effect on the CL index and C3

The CL index data are shown in Fig. 1. In the 10 mg/kg dog group, the CL index increased significantly by 1.5-fold over the Pre level on day 3 ($p < 0.01$) and then decreased to the Pre level on day 6. However, in the 10 mg/kg mouse group, the CL index decreased significantly to 0.6 times the Pre level on day 3 ($p < 0.01$) and then increased to the Pre level on day 6. On the other hand, in the 50 mg/kg mouse group, the CL index increased significantly to 1.4 times the Pre level ($p < 0.01$).

The C3 data are shown in Fig. 2. In the 10 mg/kg dog group, the C3 level increased significantly to 1.6 times the Pre level on day 3 ($p < 0.01$) and was still 1.4 times the Pre level on day 6 ($p < 0.01$ versus the Pre level). Even in the 10 mg/kg mouse group, the C3 level increased significantly to 1.3 times the Pre level on day 3 ($p < 0.05$) and then decreased to 1.2 times on day 6. In contrast, in the 50 mg/kg mouse group, the C3 level increased significantly to 1.6 times the Pre level ($p < 0.01$) on day 3 and then decreased to 1.3 times on day 6.

4.2. Effect of chitin and chitosan on C3, C4 and C5

The results of the in vitro study on C3, C4 and C5 are shown in Table 1. The positive control, zyntosan, and chitosan induced the same acute decrease of the C3 level, but chitin induced a slighter decrease. After 10 and 40 min of

Table 1
Complement activation by chitin, chitosan, and zymosan

Complement	Agent	Incubation time			
		10	20	30	40
C3	Chitosan	63 ± 02**	60 ± 05**	59 ± 04**	58 ± 06**
	Chitin	83 ± 11*	77 ± 10**	73 ± 13**	63 ± 21**
	Zymosan	66 ± 03**	64 ± 04**	64 ± 04**	64 ± 04**
C4	Chitosan	100 ± 02	100 ± 02	100 ± 02	100 ± 02
	Chitin	100 ± 02	100 ± 02	100 ± 02	100 ± 02
	Zymosan	100 ± 02	100 ± 02	100 ± 02	100 ± 02
C5	Chitosan	56 ± 19**	23 ± 32**	23 ± 32**	23 ± 32**
	Chitin	73 ± 04**	45 ± 04**	45 ± 04**	45 ± 04**
	Zymosan	56 ± 19**	23 ± 32**	23 ± 32**	23 ± 32**

The concentration of each agent is 10 mg/ml. Data were expressed as the mean % and standard deviation in each agent. 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 (*: $p < 0.05$; **: $p < 0.01$).

incubation of serum with 10 mg/ml of chitosan, C3 was decreased significantly to 63% and 58% of the Pre level ($p < 0.05$), respectively. After 40 min of incubation with chitin, C3 had decreased to 63% of the Pre level.

Zymosan, chitosan, and chitin did not have any effect on the C4 level during 40 min of incubation.

Zymosan and chitosan (10 mg/ml) decreased the serum C5 level to 56% and 23% of the Pre level after 10 and 20 min of incubation, respectively. Chitin (10 mg/kg) caused a significant decrease of C5 to 73% ($p < 0.05$) and 45% ($p < 0.01$) of the Pre level after 10 and 20 min of incubation, respectively. When serum was incubated with 1 mg/ml chitosan for 20 or 40 min, the C5 level decreased to 72% ($p < 0.05$) and 50% ($p < 0.05$) of the Pre level, respectively. Zymosan caused a 54% decrease after 40 min of incubation. Chitin caused a decrease of C5 to 87% of the Pre level after 20 min of incubation, but caused no further decrease after 40 min. Furthermore, 0.1 mg/ml of chitosan caused a decrease of C5 to 70% of the Pre level after 40 min of incubation ($p < 0.01$), while zymosan and chitin decreased the level to 70% and 90% after 40 min, respectively.

5. Discussion

We previously demonstrated that inflammatory cells migrated towards subcutaneously administered chitin or chitosan (Minami et al., 1993), and that chemotactic substances were created in serum by incubation with chitin or chitosan (Usami et al., 1997). These effects suggested humoral agents in body fluids such as serum and and interstitial fluid played a very important role in the response to chitin and chitosan. It is well known that anaphylatoxins such as C3a and C5a are created by complement activation through the alternative pathway (Law and Reid, 1995). C3a stimulates subcutaneous mast cells to produce histamine (Mousli et al., 1992; el-Lati et al., 1994; Legler et al., 1996) and leukotriene B4 (Soter, 1991; Kanai et al., 1995), and causes intensive dilation of peripheral vessels and edema. C5a stimulates phagocytes to upregulate endothelial adhesion receptor expression (Tonnesen et al., 1984). C5a also induces upregulation of the FC receptor for antibodies, and upregulation of the complement receptors CR1 and CR3, which act as ligands for C3b and iC3b, respectively (Law and Reid, 1995). In the present study, the serum C3 level and CL index were increased by subcutaneous administration of chitin and chitosan. It is known that zymosan has a C3b receptor (Super et al., 1990; Vaporciyan and Ward, 1993; Hazeki et al., 1994), since PMN activated by chitosan or chitin show increased zymosan adhesion and a stronger chemiluminescence reaction. Further, production of complement is stimulated through the activation of phagocytes by various bacteriotoxins, and by consumption of complement (von-Allmen et al., 1990; Hiyama et al., 1991; Law and Reid, 1995). When chitin and chitosan are administered subcutaneously the present in vitro study suggested that subcutaneous complement will be rapidly consumed by contact with these materials, since the increased serum C3 level in chitosan treated dogs and mice can be explained by this mechanism.

When chitosan and chitin were added to human serum, C3 and C5 decreased while C4 did not change. It is known that polyglucans such as zymosan induce the alternative pathway, and the present findings with zymosan agree well with previous reports (Zwirner et al., 1995; Seghaye et al., 1996). There is no doubt that chitosan and chitin also induces activation of the alternative pathway. From the present results, chitosan and zymosan show almost the same intensity of complement activation, while chitin has a weaker effect on complement. In the alternative pathway, C3b is created by C3i enzymatic degradation of C3

(Vetvicka et al., 1993; Law and Reid, 1995), but the mechanism of creation of C3i by chitosan is still unknown. Accordingly, further investigations of complement activation by chitin and chitosan are required.

We previously reported that subcutaneous administration of 200 mg/kg of chitosan induced lethal hemorrhagic pneumonia in dogs, while other animals did not develop pneumonia, and that 200 mg/kg of chitin did not cause this effect even in dogs (Minami et al., 1996). In acute pulmonary injury, such as the acute respiratory distress syndrome or reperfusion injury, there is activation of PMN induced by activated complement, especially C5a (Till et al., 1982). In the present study, C5 was activated by chitin and chitosan, but complement activation was more intense with chitosan. This is the reason why chitin did not cause pneumonia in dogs.

In the mouse, to induce the same activation of C3 and the CL index as in dogs, a five-fold higher dose of chitosan was acquired. This result means that there are differences in the sensitivity to chitosan between species.

Our findings indicate that biological activation starts via the alternative pathway for complement activation. C5a is produced by this activation and will promote PMN migration to wounds. These are well known inflammatory reactions, but there are no inflammatory symptoms, such as erythema, temperature elevation, or the formation of abscesses when the chitin or chitosan are used. Hence, chitin and chitosan are not only pro-inflammatory agents, but also have an anti-inflammatory effect. Further investigations of the mechanism of their anti-inflammatory effect are needed.

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