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Subcutaneous injected chitosan induces systemic activation in dogs

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Systemic activation by subcutaneous administration of chitin, chitosan, and chitosan oligomer was investigated in dogs by determining the chemiluminescence (CL) response of circulating polymorphonuclear cells (PMN). Chitin (10 and 100 mg/kg), chitosan oligomer (10 mg/kg), latex beads (10 mg/kg) and physiological saline (10 ml) did not cause activation of PMN. However, chitosan caused systemic activation of PMN in a dose-dependent manner. The peak CL response of PMN was significantly increased in the 1 and 10 mg/kg chitosan groups at 3 days after administration. In addition, chitosan (10 mg/kg) prevented a decrease of the CL response in dogs given dexamethasone. Serum obtained from the 10 mg/kg chitosan group significantly activated PMN from normal dogs. At 3 days after chitosan administration, the serum level of complement component 3 (C3) was increased about 1.6 times that of the prechitosan C3 level. In conclusion, subcutaneous administration of chitosan induces systemic activation of both PMN and serum. Copyright © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

In recent years, it has been reported that modified chitins, such as sulfated or carboxymethylated chitin, partially deacetylated chitin, and chitin oligosaccharides, show antitumor activity (Tokoro *et al.*, 1988), have an adjuvant effect (Nishimura *et al.*, 1985), and increase host resistance against bacteria (Iida *et al.*, 1987), viruses (Azuma *et al.*, 1988), and fungi (Suzuki *et al.*, 1984) when administered to experimental animals. It has also been demonstrated that unmodified chitin and chitosan promote wound healing in various animals (Minami *et al.*, 1993; Okamoto *et al.*, 1993), and activation of polymorphonuclear cells (PMN) by these materials has been reported (Minami *et al.*, 1993; Usami *et al.*, 1994a; Usami *et al.*, 1994b). These effects of chitin and chitosan can be categorized as local effects. The systemic effect of these materials has also been investigated and a decrease of cholesterol and triglyceride levels in the serum and liver has been demonstrated with oral administration of chitin to rats (Sugano *et al.*, 1978; Sugano *et al.*, 1980). Furthermore, circulating PMN are activated by intraperitoneal administration of chitin oligosaccharide in mice (Suzuki *et al.*, 1986). However, there are no data on systemic activation by chitin or chitosan in larger animals.

In the present study, the systemic effect of subcutaneous administration of chitin and chitosan was investigated in dogs by determining the luminol-dependent chemiluminescence response of peripheral PMN and the serum level of complement component 3 (C3). The effect of chitosan on PMN was also investigated in immunosuppressed animals.

EXPERIMENTAL

Drugs

Chitin and chitosan suspensions

Commercial squid pen β-chitin (Nippon Suisan Co. Ltd, Tokyo,) was purified from Neon flying squids (*Ommastrephes bartrami*); it was 9% deacetylated and had an average molecular weight of over 100 000. Chitosan flake (Flonac C, Kyowa Tecnos Co. Ltd, Japan) was also used, which comprised 82% deacetylated α-chitin purified from crab shell and had

an average molecular weight of 80000 (maximum 1.2% ash and maximum 5 ppm of heavy metals like Pb, Cd and As). These preparations were pulverized into 3 μm particles using a mill (Ube Industries Ltd, Japan, CF-400). The resulting fine powders were sterilized with ethylene oxide gas, and were suspended in physiological saline at the concentration of 30 mg/ml. Dogs were administered 10 ml of various dilutions of this suspension with physiological saline. In the 100 mg/kg chitin group, however, the chitin powder was suspended in saline at the concentration of 100 mg/ml in order to achieve the same injection volume (10 ml).

Chitosan oligomer
A chitosan oligomer mixture was purchased from Yaizu Suisankagaku (Yaizu, Japan). This mixture was deacetylated chitin oligomer obtained by depolymerization and partial acid hydrolysis of chitin prepared from snow crab shell. The chain length of D-glucosamine in the chitosan oligomer was GlcN6, and small amounts of GlcN1-GlcN5 were also present.

Latex suspension
Latex particles (polybead latex micro particles, 04-0171-34, particle size 3 μm) were purchased from Funakoshi Co. Ltd (Tokyo) and were suspended in physiological saline at a concentration of 10 mg/ml. Then an aliquot of this suspension diluted up to 10 ml with physiological saline was given to each dog.

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

Luminol solution
Luminol was diluted to 2 mg/ml using HEPES-HBSS and then 50 μl of triethylamine (Wako, Tokyo) was added to this solution. After 45 min of ultrasonication, the solution was filtered through a 0.45 μm Millipore filter in order to remove insoluble Luminol particles.

Ammonium chloride solution
Ammonium chloride (Nacalai Tesque Inc., Kyoto) and bicarbonate (Nacalai Tesque Inc., Kyoto) were added at 4.16 g and 0.42 g, respectively, to 500 ml of distilled water. Sterilization was performed by Millipore filtration.

Ficoll–Conray solution
Ficoll–Conray solution was prepared by mixing 9.5% w/v Ficoll (Pharmacia Biotech, Sweden) with 50% v/v Conray (Conray 400, Daiichi Seiyaku, Tokyo) in saline to make a solution with a specific density of 1.082.

Dexamethasone
Dexamethasone was purchased from Nihon-zenyaku (Water Soluble Dexamethasone Injection A, Fukui, Japan).

Anti-C3 serum
One mg of anti-dog C3 goat serum (Bethyl Laboratories Inc., USA) was dissolved in 2 ml of physical saline and was stored in −80°C before use.

Phosphate buffer solution
57.8 g of disodium hydrogenpliosphate 12 water (Wako Pure Chemical Industries Ltd, Osaka) and 5.92 g of disodium hydrogenphosphate dehydrate (Wako Pure Chemical Industries Ltd, Osaka) were dissolved in an adequate volume of distilled water and then this solution was diluted up to 1 liter with distilled water.

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

Animals

Forty-five adult mongrel dogs aged 1–3 years and weighing 5–15 kg, which were normal on physical and laboratory examination, were used in this study. Twenty-four dogs were used to assess the chemiluminescence response of peripheral PMN after subcutaneous administration of each agent, 17 dogs were used to study the effect of chitosan in animals on immunosuppressive therapy, and four dogs were used for the collection of normal PMN and serum.

Experiment 1
To determine the systemic effect of chitin and chitosan on normal dogs, the animals were divided into four groups: a chitin group ($n=6$), a chitosan group ($n=9$), a latex group ($n=3$), and a control group ($n=3$). The chitin group was divided into two subgroups of three dogs each which received chitin at 10 mg/kg (chitin-10) and 100 mg/kg (chitin-100). The chitosan group was divided into three subgroups of three dogs each which received chitosan at 0.1 mg/kg (chitosan-0.1), 1 mg/kg (chitosan-1) and 10 mg/kg (chitosan-10).

Experiment 2
To determine the systemic effect of chitosan oligomer on normal dogs, a chitosan oligomer solution (10 mg/kg) was administered subcutaneously to three dogs.

Experiment 3
To determine the effect of chitosan on peripheral blood parameters in detail, PMN and serum were obtained

separately from dogs in the chitosan-10 group (experiment 1). In addition, four normal healthy dogs were used for collecting blood from the jugular vein in order to separate normal PMN and serum.

Experiment 4
To determine the systemic effect of chitosan on dogs with immunosuppression, the animals were divided into four groups by the timing of chitosan and dexamethasone administration. Dexamethasone was administered before chitosan in five dogs (chitosan (−1) group), dexamethasone and chitosan were administered simultaneously in three dogs (chitosan (0) group), dexamethasone was administered after chitosan in four dogs (chitosan (+1) group), and dexamethasone was administered without chitosan in five dogs (control group). Chitosan was administered at 10 mg/kg and dexamethasone was administered at 1 mg/kg subcutaneously.

METHODS

Blood collection and examination

Three ml of blood was collected from the jugular vein with heparin (10 IU/ml) and the examinations described below were performed using 1 ml while the remaining blood was used for the chemiluminescence (CL) assay. The red blood cell count (RBC), white blood cell count (WBC), differential WBC, serum total protein (TP), and hemoglobin concentration (Hb) were measured in a routine manner.

Experiments 1 and 2
Blood was collected before chitin, chitosan, and chitosan oligomer administration (pre), as well as 3 days (day 3), 6 days (day 6), and 9 days (day 9) after administration of each agent.

Experiment 3
An additional 15 ml of blood was collected on days 3 and 6 from dogs in the chitosan-10 group, and the serum was separated from 10 ml of it after centrifugation at 3000 rpm for 15 min (chitosan serum). This serum was used for the CL and C3 assays. The 5 ml remaining was used for the separation of PMN by the method reported previously (Usami *et al.*, 1994b). In brief, 5 ml of heparinized blood was diluted with 10 ml of HEPES-HBSS, and then was layered onto 15 ml of Ficoll–Conray solution and centrifuged at 2500 rpm for 30 min. After decantation of the supernatant, red blood cells were hemolyzed with 0.83% anmonium hydrochloride. After centrifugation at 1000 rpm for 10 min, the supernatant was removed and the sediment was washed twice with HEPES-HBSS. The PMN were resuspended at a concentration of 10^6 cells/ml by addition of HEPES-HBSS (PMN suspension). Normal PMN and pooled serum (normal serum) were also prepared from four control dogs in the same manner. An aliquot of the chitosan serum and the normal serum was heated at 56 °C for 30 min (treated normal serum and treated chitosan serum, respectively).

Experiment 4
Blood collection was performed before dexamethasone administration, as well as 1 day (day 1), 3 days (day 3), 6 days (day 6), and 9 days (day 9) after dexamethasone administration.

Chemiluminescence (CL) assay

Experiments 1, 3 and 4
CL was measured with a Lumat LB-9501 (Berthold Co., Germany). The method was based upon Makimura and Sawaki (1992). In brief, blood diluted fivefold with HBSS in the cuvette was incubated at 37 °C for 15 min, and then 20 μl of Luminol (2 mg/ml) was added. After incubation for 5 min more, measurement was started. The baseline value was determined for 1 min and then 50 μl of zymosan (10 mg/ml) was added to the cuvette as a stimulator. The CL response was measured for 15 min and the peak count was determined (CL value). The CL intensity per 1000 PMN (CL index) was calculated from the following formula:

$$\text{CL index} = \{\text{CL value} \times (1 - \text{baseline index})/(G \times V)\} \times 1000.$$

Baseline index = baseline value / CL value, G = number of PMN per ml, V = whole blood volume (100 μl).

Experiment 3
500 μl of PMN suspension was incubated after addition of 500 μl of previously prepared serum (normal serum, chitosan-10 serum, normal treated, or chitosan treated serum) or HBSS (serum-free) at 37 °C for 15 min, and then 20 μl of Luminol (2 mg/ml) was added to the cuvette. The following procedures were the same as described before.

Complement component 3 (C3) estimation

C3 was assayed by the single radial immunodiffusion method. Anti-dog C3 serum was diluted twentyfold with agarose gel previously warmed at 50 °C and then was cooled in ice water. A hole was created in the center of the coagulated agar with a small corkscrew. The test serum volume was 2.5 μl and it was placed in the central hole of the agar. The incubation time was 48 h at 4 °C.

Statistical analysis

Statistical analysis was performed by the paired t test with Stat View 4.0 software or by the unpaired t test with DA stat software for Macintosh.

RESULTS

Systemic effect of chitin and chitosan after subcutaneous administration

The data on PMN are shown in Fig. 1 and Fig. 2. In the chitin-100 and chitosan-10 groups, PMN increased slightly on day 6 and days 6–9, respectively. In the latex and control groups, PMN did not change during the experimental period. The values of TP, RBC, and Hb did not change in all groups.

The CL value and CL index data for the chitin groups are shown in Fig. 3 and Fig. 4, respectively. Both parameters did not change in all of the chitin groups. On the other hand, in the chitosan-1 and chitosan-10 groups, the CL value increased significantly on day 3 ($P<0.05$) (Fig. 5). In the chitosan-10 group, the CL index also increased significantly to approximately three times the level on day 0 ($P<0.05$) (Fig. 6) and remained elevated despite a gradual decrease. In the chitosan-1 group, changes in the CL index were not significant, although day 3 and day 6 values were 1.5 and 1.7 times the day 0 value, respectively. In the latex and control groups, these values did not change (Fig. 5 and Fig. 6).

There were no significant changes in the values of RBC, Hb, and TP during experimental period in all groups.

Systemic effect of chitosan oligomers after subcutaneous administration

The results are shown in Table 1 and Fig. 7. There were no significant changes in PMN and CL index after subcutaneous administration of chitosan oligomers.

Effect of subcutaneous administration of chitosan on PMN and serum

The results are shown in Fig. 8 and Fig. 9. The CL index of normal PMN with chitosan serum, chitosan PMN with normal serum, and chitosan PMN with chitosan serum was increased by factors of 1.74, 1.35, and 1.61, respectively, with respect to the value for normal PMN with normal serum (control). There were significant increases in the CL index compared to the control. The CL index of the serum-free assay (0.32 times) was significantly decreased ($P<0.01$) compared to the control.

The effect of heat treatment of serum is shown in Fig. 9. The CL index of treated chitosan serum was 49% lower than that of untreated chitosan serum ($P<0.01$), and that of treated normal serum was decreased 53% ($P<0.01$). However, the CL index of treated chitosan serum was significantly higher than that of treated normal serum ($P<0.01$).

Changes in the C3 level after chitosan administration (10 mg/kg) are shown in Table 2. The C3 level increased significantly on days 3 and 6 ($P<0.01$).

Systemic response to chitosan administration in immunosuppressed dogs

The WBC count data are shown in Fig. 10. In the dexamethasone group (control), the WBC count increased 150% on day 1 (100% = day 0), but on day 3 it returned to the day 0 level. In the three chitosan groups, the WBC count increased rapidly and decreased gradually, being maintained at 130% even on day 9.

CL index data are shown in Fig. 11. In the dexamethasone group, the CL index decreased by 67% relative to day 0, while the chitosan groups only showed decreases of 15–35%.

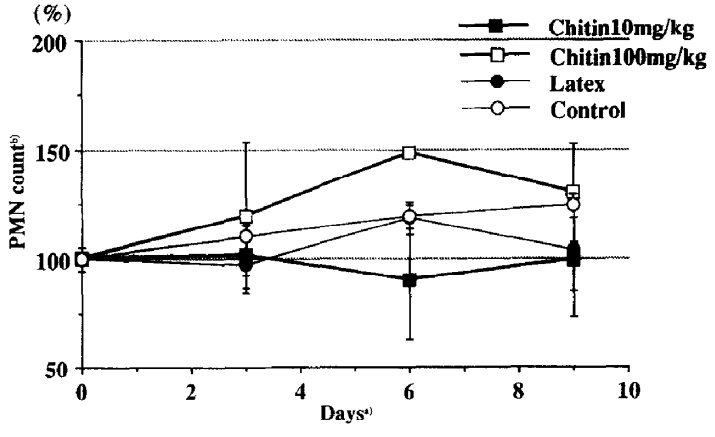

Fig. 1. Variation in PMN counts in chitin groups. a) before injection, PMN count was examined at 0, 3, 6 and 9 days after injection. b) PMN count was expressed in mean % and standard error bar in each group. Individual data were converted to % by each initial PMN count (day 0).

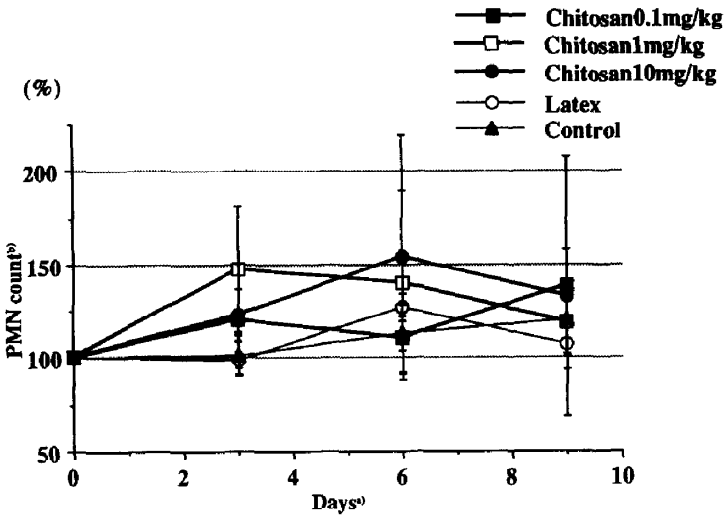

Fig. 2. Variation in PMN counts in chitosan groups. a) before injection, PMN count was examined at 0, 3, 6 and 9 days after injection. b) PMN count was expressed in mean % and standard error bar in each group. Individual data were converted to % by each initial PMN count (day 0).

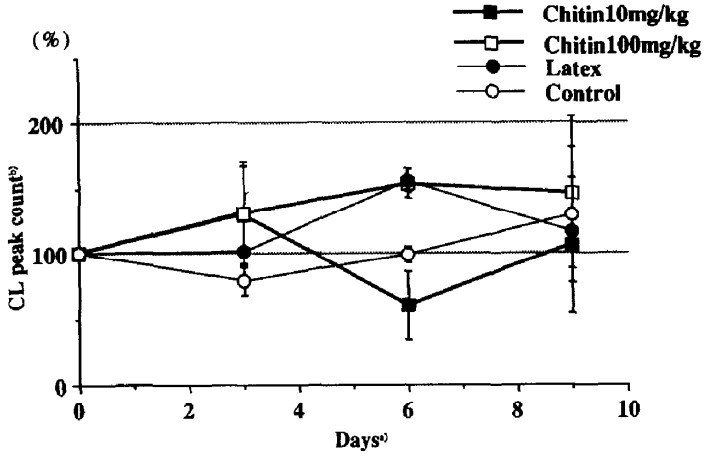

Fig. 3. Variation in CL values in chitin groups. a) before injection, CL was measured at 0, 3, 6 and 9 days after injection. b) CL values were expressed in mean % and standard error bar in each group. Individual data were converted to % by each initial CL peak count (day 0).

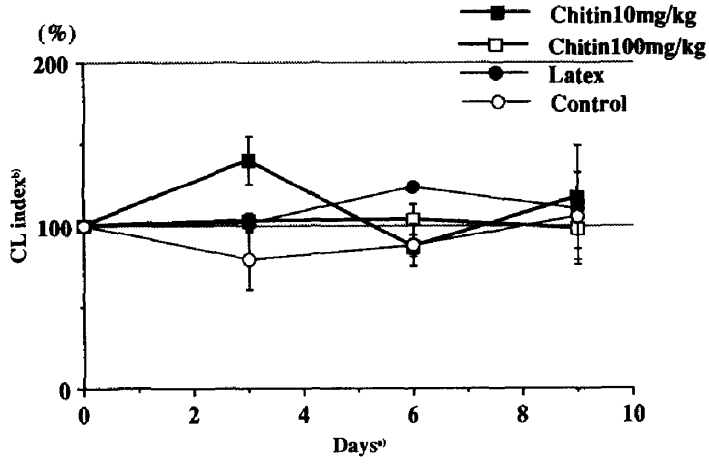

Fig. 4. Variation in CL indexes in chitin groups. a) before injection, CL was measured at 0, 3, 6 and 9 days after injection. b) CL indexes were expressed in mean % and standard error bar in each group. Individual data were converted to % by each initial CL index value (day 0).

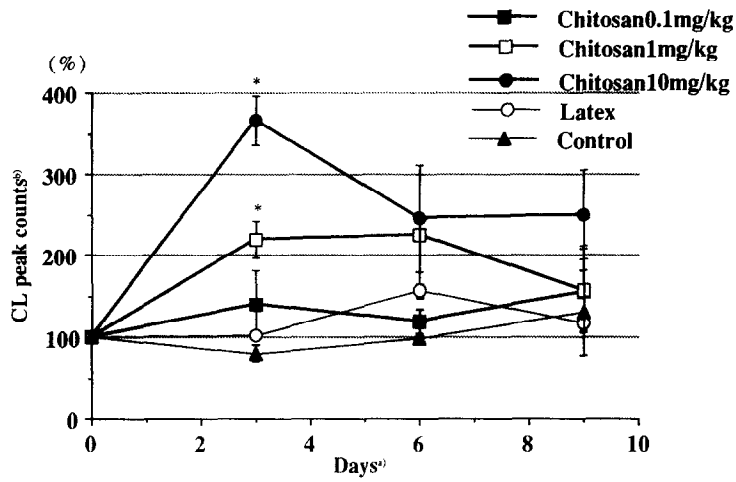

Fig. 5. Variation in CL values in chitosan groups. a) before injection, CL was measured at 0, 3, 6 and 9 days after injection. b) CL values were expressed in mean % and standard error bar in each group. Individual data were converted to % by each initial CL index value (day 0). * Values are significantly different between control and chitosan groups ($P < 0.05$).

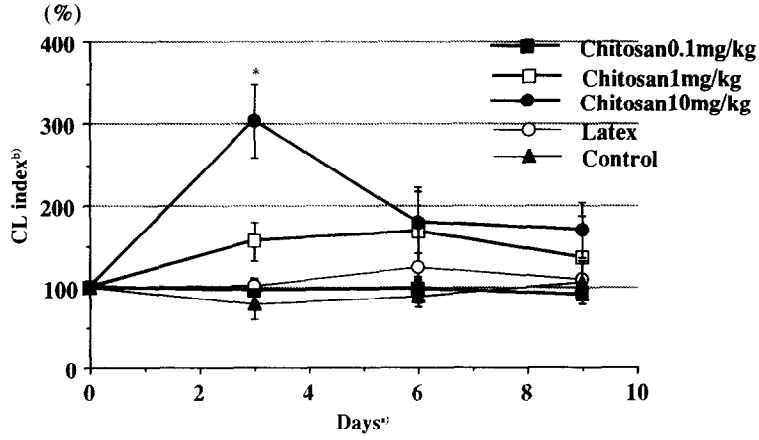

Fig. 6. Variation in CL indexes in chitosan groups. a) before injection, CL was measured at 0, 3, 6 and 9 days after injection. b) CL indexes were expressed in mean % and standard error bar in each group. Individual data were converted to % by each initial CL index value (day 0). * Values are significantly different between control and chitosan 10 mg/kg ($P < 0.05$).

Table 1. Systemic effect of chitosan oligomer by subcutaneous administration

WBC and CL	Days after administration of chitosan oligomer			
	0[a]	3	6	9
WBC[b]	14167	13167	11267	11167
s.e.[c]	2827	1172	1314	1365
PMN[d]	7902	11454	8396	9299
s.e.	1334	1509	625	1118
Total CL[e]	15385	23876	20568	21143
s.e.	3611	6562	2728	2556
CL index[f]	1913	1960	2420	2273
s.e.	303	365	157	17

[a]Before injection. Each parameter was then examined at 3, 6 and 9 days after injection.
[b]White blood cell count per 1 cm^3 of blood.
[c]Standard error.
[d]Polymorphonuclear cell count per 1 cm^3 of blood.
[e]Total chemiluminescence (CL) counts. Values are expressed in relative light units.
[f]CL counts of each 1000 polynuclear cells. Values are expressed in relative light units.

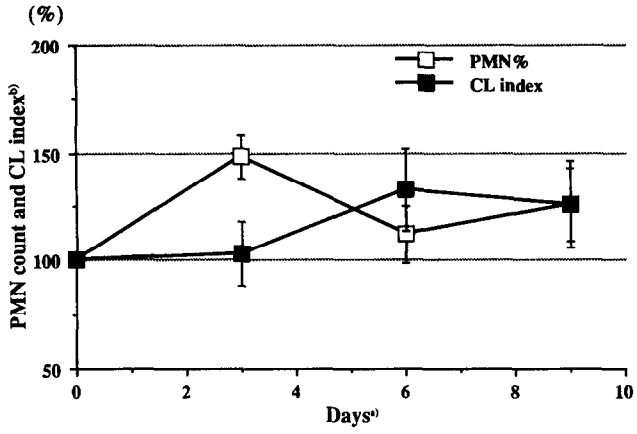

Fig. 7. Variation in PMN counts and CL index in chitosan oligomer group. a) before injection, PMN count was measured at 0, 3, 6 and 9 days after injection. b) PMN count and CL index were expressed in mean % and standard error bar in each group. Individual data were converted to % by each initial PMN count and CL index (day 0).

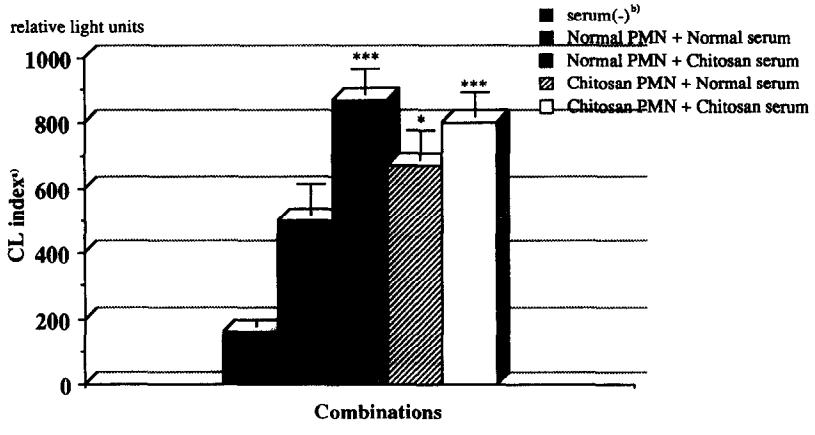

Fig. 8. Influence of subcutaneous administered chitosans on circulatory PMN and serum. a) CL peak counts (CL) in various sample combinations. b) Combinations are as follows: serum(−):CL of normal PMN separated in HBSS from non-chitosan injected dog under incubation with zymosan; normal PMN + normal serum:CL of normal PMN under incubation with zymosan and normal serum; normal PMN + chitosan serum:CL of normal PMN under incubation with zymosan and serum which was separated from chitosan injected dog; chitosan PMN + normal serum:CL of PMN which were separated from chitosan injected dog under incubation with normal serum; chitosan PMN + chitosan serum:CL of chitosan PMN under incubation with zymosan and chitosan serum; ***, *: values are significantly different between serum(−) and other combination (***$p < 0.01$, *$p < 0.05$).

DISCUSSION

Subcutaneous administration of chitosan induces the activation of PMN and the intensity of PMN activation is dose dependent. Suzuki *et al.* (1984) reported that cell numbers, superoxide production, and the CL response of peripheral PMN and peritoneal exudate cells (PEC) were increased by chitin at a dosage of 50 mg/kg administered intraperitoneally in mice, but those effects were not observed with chitosan. Also, when PEC were induced by administration of various chain length chitin oligosaccharides, the cells were most activated by *N*-acetyl-chito-oligosaccharide 6 (Suzuki *et al.*, 1986). In the present study, PMN were more activated by the administration of chitosan than by chitin. These findings are different to those of Suzuki *et al.* (Suzuki *et al.*, 1984, 1986), but it is difficult to compare them because the route of administration, the experimental animals, and the CL stimulators were completely different in the two studies. These saccharides may induce systemic activation of immune cells in animals. In the present study, activation of PMN and an increase of serum C3 by chitosan were clearly demonstrated. The PMN and serum recovered on day 3 from chitosan-treated dogs were 35% and 74% more activated than normal, respectively. It was clearly demonstrated that subcutaneous injection of chitosan induced more biological activation of serum components than of the circulating cells.

PMN are activated by infections and inflammatory reactions, after which they migrate to the inflammatory

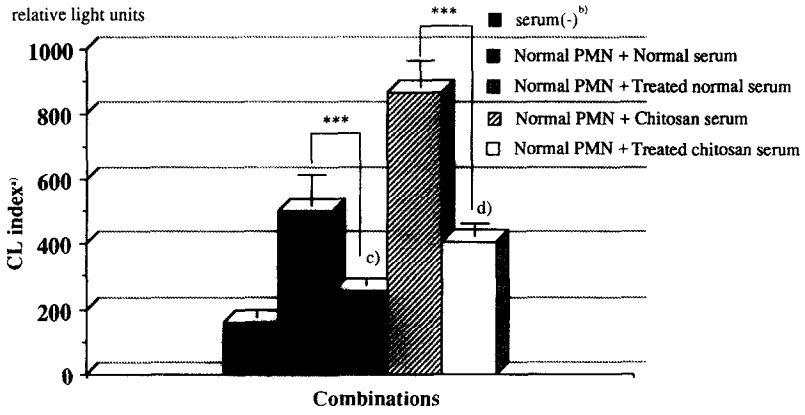

Fig. 9. Influence of subcutaneous administered chitosan on circulatory PMN and serum. a) CL peak counts (CL) in various sample combinations. b) Combinations are as follows:serum(−):CL of normal PMN separated in HBSS from non-chitosan injected dog under incubation with zymosan; normal PMN + normal serum:CL of normal PMN under incubation with zymosan and normal serum; normal PMN + treated normal serum:CL of normal PMN under incubation with zymosan and heat treated normal serum (56°C, 30 min); normal PMN + chitosan serum:CL of normal PMN under incubation with serum which was separated from chitosan injected dog; normal PMN + treated chitosan serum:CL of normal PMN under incubation with zymosan and heat treated chitosan serum (56°C, 30 min); ***: values are significantly different ($P < 0.01$), c) and d) are also significantly different ($P < 0.01$).

Table 2. Variation of C3 level in canine serum and CL index of PMN after 10 mg/kg chitosan subcutaneous administration

C3 and CL index	Days after administration of chitosan		
	0[a]	3	6
C3[b]	100	159*	141*
s.e.[c]		8	9
CL index[d]	100	300**	180
s.e.		50	45

[a]Before injection.
[b]C3 levels are expressed as mean %. Individual data were converted to % by each initial C3 level (day 0).
[c]Standard error.
[d]CL indexes were expressed as mean %. Individual data were converted to % by each initial CL index (day 0).
*$P < 0.01$.
**$P < 0.05$.

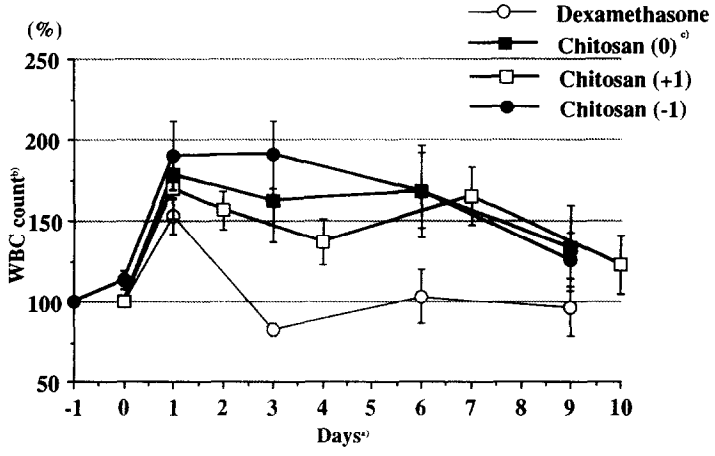

Fig. 10. Variation in WBC counts in chitosan group under the immuno-suppression with dexamethasone (dexa) injection; a) 0 days, dexa was injected (1 mg/kg); b) All WBC counts were converted to % by each initial WBC count before injection; c) chitosan (0): 10 mg/kg was simultaneously injected with the dexa; chitosan (-1): chitosan was injected 1 day before the dexa injection; chitosan (+1): chitosan was injected 1 day after the dexa injection.

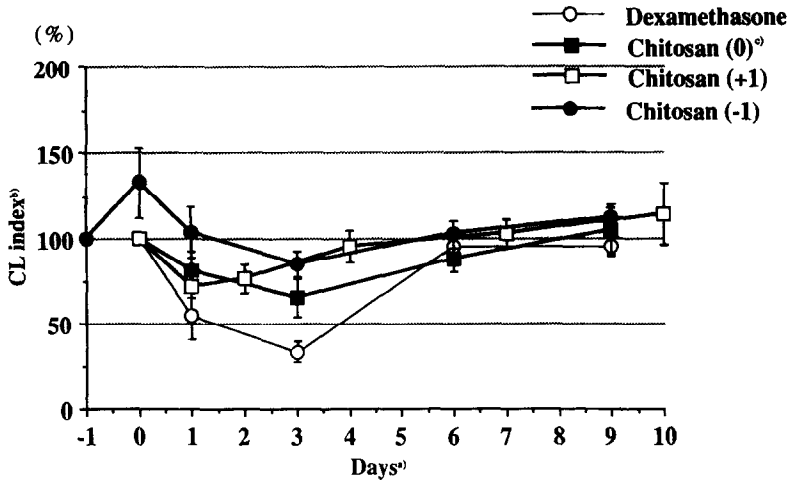

Fig. 11. Variation in CL indexes in chitosan groups under the immuno-suppression with dexamethasone (dexa) injection; a) 0 days, dexa was injected (1 mg/kg); b) All CL indexes were converted to % by each initial CL index before injection; c) chitosan (0): 10 mg/kg was simultaneously injected with the dexa; chitosan (-1): chitosan was injected 1 day before the dexa injection; chitosan (+1): chitosan was injected 1 day after the dexa injection.

site to perform phagocytosis and bactericidal activities (Minato, 1992). Activation of phagocytosis is induced by immune complexes, C5a, LTB4, platelet activating factor, and opsonization of foreign bodies (Takano and Mineo, 1990). In the present study, C3 was significantly increased by 10 mg/kg of chitosan and the result of the serum activation study would suggest that opsonization of zymosan had occurred. Zymosan has CR3 receptors on the cellular surface and CR3 interact with C3bi (Ross *et al.*, 1985; Hazeki *et al.*, 1994). C3bi receptors also exist on the surface of PMN (Ross *et al.*, 1985; Hazeki *et al.*, 1994). These effects were induced by the activation of the complement, since chitosan serum showed decreased activation after heat treatment. It is well known that serum complement is decomposed by this treatment, although heat-resistant complement components such as C5a will remain (Honda and Hayashi, 1982). In the present study, chitosan oligomer did not influence the PMN count and CL index. It is known that chitosan oligomers have a strong ability to promote PMN migration after subcutaneous injection in dogs, but do not induce complement activation (Usami *et al.*, 1997).

Chitosan and chitin are water-insoluble materials, so a direct physical effect may be possible. However, water-insoluble latex particles with a similar size to the chitosan particles did not induce these effects. This suggests that the chemical and/or molecular structure of chitosan was related to its effects.

In this experiment, chitin did not induce similar activation to chitosan even at a dose of 100 mg/kg, which was 10 to 100 times the dose at which chitosan induced PMN activation. Chitin and chitosan have similar chemical structures, and the difference lies in the number of D-glucosamine or *N*-acetyl-D-glucosamine residues. Chitosan has about eight times the D-glucosamine found in chitin. However, the present results cannot be explained by the differences in D-glucosamine residues, because the number of D-glucosamine residues in a dose of 100 mg/kg of chitin is about ten times that in a dose of 1 mg/kg of chitosan.

Chitosan treatment was also effective for PMN activation in dogs with immunosuppression due to dexamethasone administration. The immuno-suppressive mechanism of dexamethasone is based on suppression of PMN functions induced by complement activation (Munoz *et al.*, 1989; Lemercier *et al.*, 1992) and on the prevention of PMN accumulation by a direct influence on these cells and on the endothelium (Yarwood *et al.*, 1993). The present results suggest that chitosan activates the complement more efficiently and continuously than dexamethasone blocks complement activation.

From these findings, it is possible that preoperative administration of chitosan will induce host defenses against various surgical and/or anesthetic stresses. However, further investigation is required about its effect on complement activation and on PMN dysfunction induced by surgical procedures.

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