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Protective effects of phosphated chitin (P-chitin) in a mice model of acute respiratory distress syndrome (ARDS)

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Abstract

This study has investigated the potential usefulness of phosphated chitin (P-chitin) as an anti-inflammatory agent in a mice model of chitosan-induced acute respiratory distress syndrome. A unique model of interstitial pneumonia that mimics acute respiratory distress syndrome was developed by an overdose of an intraperitoneal injection of chitosan suspension at the rate of 1–4 g/kg body weight over a period of 24–48 h. Pneumonia thus induced was blocked by a simultaneous intravenous injection of P-chitin at the rate of 8–40 mg/kg body weight. The protective effects of P-chitin were evaluated, based mainly on the computerized image analysis of the histological lung sections, for the degree of infiltration in the interstitial and alveolar spaces by the neutrophils and other cellular and non-cellular elements. Intravenous infusion of some P-chitin formulations dramatically reduced lung injury and diminished the accumulation of neutrophils in the interstitial and alveolar spaces of the lungs, whereas other P-chitin had no such effects. Of the nine types of P-chitin tested, only three P-chitin formulations were significantly effective in the prevention of pneumonia. P-chitin with a molecular weight of 24 000, the degree of substitution at 58%, and the degree of deacetylation at 4% was found to be most effective in blocking the chitosan induced lung injury when administered at 8 mg/kg level. The degree of blockage of pneumonia by P-chitin was comparable (significant at $p < 0.05$ level) to that of control (only chitosan). The present results suggest that P-chitin may be used as a novel anti-inflammatory agent. The possible mechanism of action for this phenomenon is under investigation.

Keywords: Phosphated chitin (P-chitin); Chitosan; Pneumonia; Mice; Acute respiratory distress syndrome (ARDS); Degree of deacetylation (DDA); Degree of substitution (DS)

1. Introduction

Many beneficial effects of chitin, chitosan and their derivatives in animal and human wound management have been published (Kifune, 1992; Minami et al., 1992, 1993; Okamoto et al., 1992, 1993; Okamoto, Shibazaki, Minami, Matsuhashi, Tanioka & Shigemasa, 1995). Chitin, chitosan and their derivatives have been reported to exhibit antitumor activity (Tokoro, Tatewaki, Suzuki, Mikami, Suzuki & Suzuki, 1988), an adjuvant activity (Nishimura, Nishimura, Seo, Nishi, Tokura & Azuma, 1986), and an increased host resistance against bacterial, viral, and fungal infections (Iida et al., 1987; Suzuki, Okawa, Hashimoto, Suzuki & Suzuki, 1984) in experimental animals. However, Minami et al. (1996) found the development of lethal pneumonia in 70% of the dogs that were injected subcutaneously with a very high dose of chitosan at the rate of 200 mg/kg body weight. Histopathological findings reportedly revealed that chitosan induced pneumonia resulted in the infiltration of polymorphonuclear (PMN) cells and severe hemorrahaging in the interstitial spaces of the lung. Likewise, high doses of chitosan was reported to induce pneumonia in mice (Takayama, 1996) closely mimicking acute respiratory syndrome distress (ARDS). Since these findings in dogs and mice are closely resembling ARDS (Lesur et al., 1999; Mulligan, Paulson, De Frees, Zheng, Lowe & Ward, 1993; Murray, 1974), we decided to use the chitosan-induced ARDS model in mice to evaluate the different P-chitin formulations for their anti-inflammatory activity.

Despite the present day advanced treatment regimen and quality medical care and management of the patients, mortality rates for ARDS have remained relatively constant; though this syndrome has been well described about 30 years ago. Various synthetic steroidal and non-steroidal anti-inflammatory agents are being used with varying degrees of success for the management of ARDS patients.

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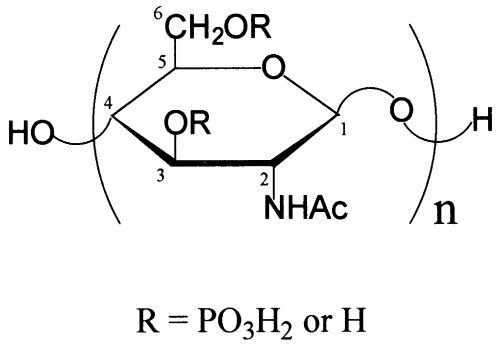


Fig. 1. Chemical structure of P-chitin.

In addition, these synthetic agents also possess some side effects such as gastric irritation, ulceration, decreased host resistance, etc. in the patients. In order to find some natural compounds that have anti-inflammatory activity and at the same time are not recognized as the foreign materials in vivo due to their biocompatibility, and biodegradability, extensive studies have been undertaken with chitin, chitosan and their derivatives, in recent years. Muzzarrelli (1988) briefly cited that carboxy methyl chitin (CM chitin) was effective for inducing cytotoxic macrophages whereas phosphorylated and sulphonated chitin was potent cytostatic and induced H_2O_2 releasing activity in macrophages. After that, no works were reported on the biomedical activity of phosphated chitin (P-chitin) until very recently, when we speculated on its anti-inflammatory property due to its anionic nature and possession of heparinoid-like activity imparted from phosphate moieties at 3 and 6-OH positions in the chitin skeleton. Hence, it was presumed that P-chitin might probably behave as a phosphate buffer with an additional beneficial activity in vivo. The aim of the present study was to evaluate, by means of a computerized image analysis, the degree of infiltration in the interstitial and alveolar spaces of mice lungs after simultaneous administrations of P-chitin and chitosan. In particular, we wanted to determine to what extent different P-chitin formulations would influence the histological parameters of the lungs in terms of interstitial and alveolar infiltration. Also, we wanted to find out whether the densitometric profiles of the interstitial and alveolar spaces of the lungs would vary with the amount of chitosan and P-chitin injected, besides the influence of the two different routes of administration of P-chitin.

2. Materials and methods

2.1. Animal

After 1 week of acclimatization at the laboratory environment, 5–6 weeks old ($n = 88$) female *ddy* mice weighing approximately 25 g (20–30 g) were used in this study. They were fed with commercial chow and drinking water ad lib. Animal treatments were performed on the basis of the Guidelines for Animal Experimentation of the Faculty of Agriculture of Tottori University, the Japanese Government Animal Protection and Management Law, and the Japanese Government Notification on Feeding and Safe keeping of Animals. Animals were used for the experiment after obtaining permission from the Animal Committee of the Faculty of Agriculture of Tottori University. Fifty ($n = 50$) mice were used to study the influence of three different P-chitin formulations in relation to the degree of substitution (%DS) on pneumonia blocking property (first experiment), in addition the histological changes subsequent to two different routes of P-chitin administration were also studied. The effect of administering a high dose of chitosan and the simultaneous administration of P-chitin treatment over a longer duration of time was investigated using another 20 mice (second experiment). The remaining 18 mice (third experiment) were utilized to evaluate the pneumonia blocking activity of six types of P-chitin formulations having different DS, deacetylation (DDA), and molecular weight (M_w and M_n).

2.2. Preparation of chitosan samples

Chitosan powder from snow crab shells (Sunfive Inc, Japan) with >82% DDA and particles <10 μm (mean particle size: 5 μm) was used in this study. The distribution of granule size was measured with a SK Laser Micron Sizer 7000S (Seisin K K, Japan). The endotoxin content was undetectable in the hot water extract (70°C) by the specific colorimetric examination method (Endospecy, Seikagaku-Kogyo, Japan). The chitosan powder was sterilized by ethylene oxide gas, and was suspended in sterile physiological saline before use. The chitosan suspension was prepared aseptically, in two different levels of concentrations viz. 50 and 100 mg/ml and stored in the refrigerator until its use. The pH of chitosan suspension at 25°C was 7.8.

2.3. Preparation of phosphated chitin

P-chitin was synthesized by the phosphorylation of chitin, based on the method described by Tokura and Tamura (1998). In brief, 30 g of urea was suspended in 30 ml of DMF (*N*,*N*-dimethyl formamide) and a homogenous solution was prepared by stirring at 100°C. Two grams of dried, fine chitin powder from squid pen was added to this solvent at 100°C until the added chitin was swollen. After 5.2 ml of *ortho*-phosphoric acid was added, the reaction was stirred for 3 h at 150°C. The reaction mixture was rinsed extensively by methanol (about 500 ml) until the color imparted from residual phosphoric acid and DMF disappears and the mixture becomes colorless. The residue was dissolved in water and water-soluble fraction of pH 10–11 was dialyzed against de-ionized water repeatedly to remove urea and phosphoric acid. The residue was then lyophilized and the insoluble part was separated and treated by acetone, followed by a methanol rinse and air-drying.

Table 1
Chemical characteristics of different P-chitin formulations. (Initial pH was measured at 25°C and concentration of all P-chitin solutions was 1 mg/ml)

P-chitin samples	pH	DDA[a] (%)	DS[b] (%) α[c]	M_n[d]	M_w[d]	M_w/M_n
35-3-4 (S1)	3.5	4	1.16 (58.0)	24 000	96 000	4.0
35-4-3 (S2)	3.7	4	0.64 (32.0)	27 000	110 000	4.1
36-2-1 (S3)	7.3	5	0.59 (29.5)	32 000	95 000	3.0
36-1-1 (S4)	7.4	7	0.47 (23.5)	22 000	60 000	2.7
38-3-2 (S5)	6.8	8	0.75 (37.5)	25 000	56 000	2.2
38-4-1 (S6)	7.2	0	1.16 (58.0)	39 000	49 000	1.3
36-3-1 (S7)	7.4	3	0.68 (34.0)	37 000	120 000	3.2
39-3-1 (S8)	7.0	5	1.03 (51.5)	81 000	129 000	1.6
39-2-1 (S9)	6.2	9	1.57 (78.5)	78 000	97 000	1.2

[a] DDA (degree of deacetylation): ^{1}H NMR analysis.
[b] DS (degree of substitution): elemental analysis and ^{1}H NMR analysis.
[c] α: substitution of phosphate group at 3 and 6-OH positions (values in brackets represent the percentage of substitution; maximum value of DS is 2.00, which means a 100% substitution by the phosphate group).
[d] Average molecular weight (M_w) and median molecular weight (M_n): GPC analysis.

The DDA (%) was determined by ^{1}H NMR analysis. The M_w and M_n of water soluble P-chitin was estimated by GPC (gel permeation chromatography) using Asahipak GF-1G7b, GS-510H, GS-310H and GS-220H columns (Schodex®, Asahi Chemical Industry Co Ltd, Japan) and Pullulane standard (Shodex Standard P-82®, Showa Denko K K, Japan). The flow rate of the buffer was 1 ml/min, and the column temperature was 50°C. The DS of the phosphate group at the 3- and 6-OH positions of the chitin skeleton (as shown in Fig. 1) was determined by elemental and ^{1}H NMR analyses. The formula used for the calculation of DS was:

DS = (P%/31)/(N%/14), where P% indicates percentage of phosphorous and N% indicates percentage of nitrogen. P% was determined by elemental analysis and N% by using the following formula.

N% = 14C%/12(8 − 2DDA/100), where C% indicates the percentage of carbon and DDA represents the degree of deacetylation of starting chitin sample. N% was determined by elemental analysis and DDA by NMR analysis.

Table 2
Results of the image analysis of the histological lung sections of mice in Experiment 1. (Per area infiltration was estimated by Image Pro® Plus software (Version 3.0 for Power Macintosh© 1996 Media Cybernetics) as described in the experimental methods (***$p < 0.001$; **$p < 0.01$ and *$p < 0.05$ from the positive control). Chitosan was administered at the rate of 1 g/kg (0.5 ml of 50 mg/ml suspension). Concentration of all P-chitin samples was 1 mg/ml

Mice groups	Number of animals ($n = 50$)	Per area infiltration in the interstitial spaces of the lung (mean ± std. dev.)
I. Chitosan (CTS) control (i.p.)	6	39.22 ± 3.35
II. CTS + P-chitin (i.p. and i.p.)	20	
i. S1, i.v. (8 mg/kg)	5	20.64 ± 1.12***
ii. S2, i.v. (8 mg/kg)	3	33.60 ± 6.36
iii. S3, i.v. (8 mg/kg)	3	45.11 ± 4.17
iv. S1, i.p. (20 mg/kg)	3	25.15 ± 6.68**
v. S2, i.p. (20 mg/kg)	3	37.15 ± 5.85
vi. S3, i.p. (20 mg/kg)	3	38.57 ± 7.58
III. P-chitin control (i.v. and i.p.)	18	
i. S1, i.v. (8 mg/kg)	3	27.65 ± 7.87*
ii. S2, i.v. (8 mg/kg)	3	35.02 ± 6.06
iii. S3, i.v. (8 mg/kg)	3	38.23 ± 4.54
iv. S1, i.p. (20 mg/kg)	3	27.81 ± 5.26*
v. S2, i.v. (20 mg/kg)	3	43.77 ± 3.67
vi. S3, i.v. (20 mg/kg)	3	32.87 ± 7.01
IV. Saline control (i.v. and i.p.)	6	
i. Saline, i.v. (0.2 ml)	3	28.87 ± 8.88*
ii. Saline, i.p. (0.5 ml)	3	28.18 ± 13.00*

Once the values for of N%, C%, P% and DDA were obtained, DS could be calculated easily. The maximum value of DS would be 2.00 or 100%, if all 3- and 6-OH positions were fully substituted.

The freeze-dried P-chitin was dissolved in sterile physiological saline at 1 or 5 mg/ml concentration. The pH was adjusted to blood pH (7.35–7.45), before the sterilization by 0.45 μm Millipore (Sterivex®, Millipore Corporation) filtration. After sterilization, the P-chitin sample was stored in the refrigerator until use. The chemical characteristics, and the initial pH profiles of all P-chitin samples before pH adjustment at 25°C are listed in Table 1.

2.4. Experimental trials

2.4.1. Influence of the DS of P-chitin and routes of administration on pneumonia blocking property (Experiment 1)

Fifty ($n = 50$) female, *ddy* mice were divided into four groups. The first group ($n = 6$) served as chitosan control, the second group ($n = 20$) received a simultaneous injection of chitosan and P-chitin challenge, and the third ($n = 18$) and fourth groups ($n = 6$) served as P-chitin and saline controls, respectively (Table 2). Mice from all four groups received 0.1 ml (mixture of Xylazine HCl 20 mg/ml (Selectal®, Bayer, Japan) and Ketamine HCl 50 mg/ml (Ketalar®50, Parke-Davis) in 1:8 ratio) of anesthetic by intraperitoneal (i.p.) injection before receiving any other injections. Chitosan suspension was administered at the rate of 1 g/kg body weight (0.5 ml of 50 mg/ml) by i.p. route using a 25 gauge needle, to induce pneumonia within 24 h. The observation period of 24 h for the development of discernible pneumonic lung was tentatively fixed based on the preliminary study by sacrificing mice at 0, 2, 4, 6, 8, and 24 h after the chitosan injection. Three types of P-chitin samples (S1, S2 and S3) with different DS but with almost the same DDA and Mw were injected simultaneously by either intravenous (i.v.) or i.p. routes. The volume of P-chitin (1 mg/ml) injected was 0.2 ml (at the rate of 8 mg/kg) and 0.5 ml (at the rate of 20 mg/kg) for i.p. and i.v. routes, respectively, in the treatment groups. The volumes of P-chitin and saline in control groups were the same as in their respective treatment groups. At the end of the 24 h observation, a post-mortem examination was performed, following the sacrifice of all the mice by ex-sanguination, for the macroscopic abnormalities of the lungs and the peritoneum, if any. Lung samples were collected and fixed in 10% neutral buffered formalin for 48 h, embedded in paraffin blocks, sections cut and permanent slide mounts prepared after staining with hematoxylin and eosin (H&E). Histological slides of all groups were analyzed by computer aided image analysis for the degree of infiltration of interstitial areas by inflammatory cells especially PMN cells and macrophages besides other cellular and non-cellular components.

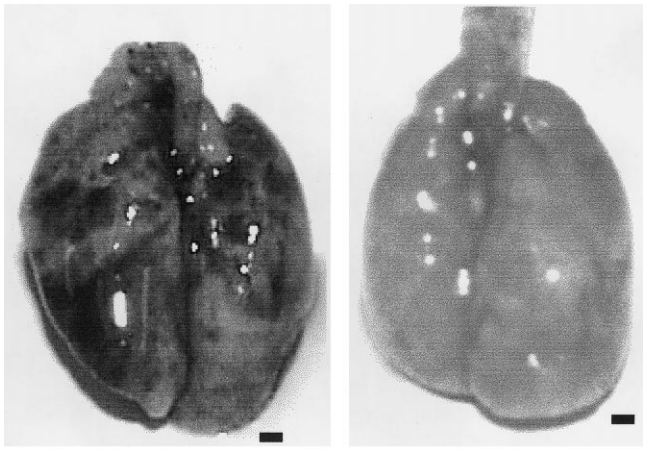
2.4.1.1. Computerized image analysis of the histological lung sections. Image analysis was performed with the use of a microscope fitted with a camera, television monitor and computer. At low power examination ($20\times = 200$ times of magnification) of the microscope, 10 microscopic fields were randomly selected and their pictures were retrieved in the computer with the help of a Fujix Digital Camera HC-300 (Fuji Photo Film Co, Ltd) and Photo Grab™-300 SH-3 software (Version 1.0 for Windows® and Macintosh, Copyright ©1997 Fuji Photo Film Co, Ltd). The images thus retrieved were converted to gray scale and then analyzed by Image Pro® Plus software (Version 3.0 for Power Macintosh© 1996 Media Cybernetics). The degree of infiltration in the interstitial spaces was determined by counting and measuring the dark objects after gray scaling and expressed as per area infiltration (ratio of object area/total area). The mean of 10 lung fields for every experimental group was recorded in the entire group and statistical analyses were performed by Student's *t*-test.

2.4.2. Effect of high doses of chitosan and simultaneous administration of P-chitin treatment over a longer duration of time (Experiment 2)

This experiment was carried out by administering higher doses of chitosan with a longer duration of time, i.e. for 48 h and with the best P-chitin selected from the previous experiment. Since clinical signs with obvious macroscopic findings suggestive of pneumonia were noticed with an extended period of observation for 48 h of chitosan challenge in another preliminary study, we decided to administer a high amount of chitosan and the best P-chitin from the first experiment and observe the outcome. Twenty ($n = 20$) female *ddy* mice of 5–6 weeks age were divided into four groups. The first group ($n = 8$) was sub-divided into two sub-groups with 5 mice in one and 3 in another, and received chitosan suspension at the rate of 2 and 4 g/kg body weight, respectively. The second group ($n = 6$) received simultaneous P-chitin injection (S1: at the rate of 40 mg/kg, i.v.) and chitosan challenge (at the rate of 2 g/kg, i.p.); the third group ($n = 3$) served as P-chitin control (S1: at the rate of 40 mg/kg, i.v.); and finally the fourth group ($n = 3$) served as saline control (0.2 ml, i.v.). All the animals were observed for 48 h. Other experimental protocols were similar to those in Experiment 1.

2.4.3. Evaluation of different P-chitin formulations (Experiment 3)

After investigating the anti-inflammatory activity of three formulations of P-chitin having different DS but almost the same DDA and M_w, we wanted to investigate further six more P-chitin (S4–S9) formulations that have different DS, M_w and DDA. A total of 18 mice (3 mice in each group) were used in this experiment. All the mice received simultaneous injections of chitosan (at the rate of 2 g/kg, i.p.) and P-chitin (at the rate of 40 mg/kg, i.v.) once as in previous experiments and were observed for 48 h after the



i. ii.

Fig. 2. The macroscopic appearance of the enlarged photographs of the lungs from mice: (i) receiving only 2 g/kg chitosan; and (ii) simultaneous intravenous P-chitin (40 mg/kg) treatment; after 48 h (postmortem findings). Scale bar (4 mm) in both cases: 1 mm, viz. magnified four times from the original photos.

injections. Other protocols for the entire experiment were similar to the previous experiments.

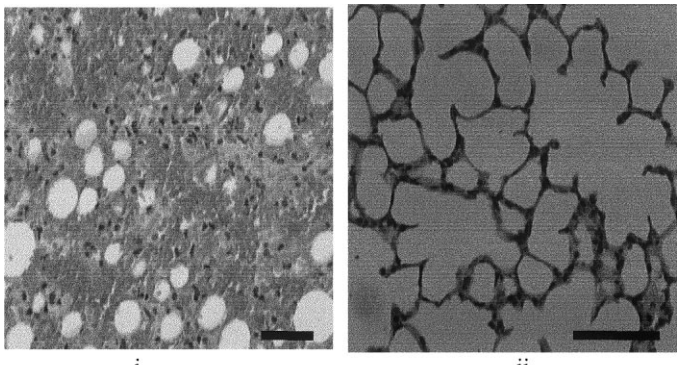
3. Results

3.1. Development of a pneumonic model (Experiments 1 and 2)

Interstitial pneumonia was produced in all the chitosan control mice and the severity of pneumonia was dose dependent. When the mice were injected with chitosan suspension at the rate of 1 g/kg by i.p. route (0.5 ml of 50 mg/ml), a less severe form of interstitial pneumonia was evident in 24 h than mice receiving chitosan at the rate of 2–4 g/kg in 48 h. The lungs at post-mortem examination were intensely pneumonic (Fig. 2i), and the cut surfaces frequently resembled the liver. There was a varying degree of peritonitis with or without pneumonia (normal) in mice receiving simultaneous injections of chitosan and P-chitin (Fig. 2ii). Mice receiving only chitosan were duller than the saline control mice or the ones that received the best P-chitin sample. Some P-chitin formulation (S3) was not able to block the pneumonia and, instead, aggravated the condition. The histology of chitosan induced pneumonic lungs in mice revealed an extensive and diffuse interstitial and alveolar infiltration by PMN leukocytes besides edema and severe hemorrhage when compared to the lungs of saline control mice and mice treated with the best P-chitin (Figs. 3 and 4). Some degree of fibrosis of alveolar walls, presence of macrophages in the alveoli, aggregates of organized fibrin in the alveoli were also observed in untreated cases. There was endothelial attachment and aggregation of PMN cells. Hyaline membrane was also evident at times in the alveoli and small airways. After image analysis of the lung sections, results were expressed as the percent of lung tissue involved in terms of the degree of infiltration in the interstitial spaces (per area).

3.2. Influence of DS of P-chitin formulations on pneumonia blocking activity (Experiment 1)

The clinical response of three different P-chitin samples was variable ranging from excellent blocking to further aggravation of the pneumonia. The response shown by P-chitin (S1), with a DS of 58%, DDA of 4% and M_n of 24 000, was found to be superior among all the three samples tested and was comparable to that of saline control. The degree of infiltration in the normal lungs was found to be around or below 30%. The response of the lung in each group of animals, expressed in terms of the degree of infiltration in the interstitial spaces is presented in Table 2. In mice receiving simultaneous chitosan challenge and P-chitin by i.p. route, the degree of infiltration was usually higher than those receiving the same P-chitin by i.v. route. In P-chitin control mice (i.v. and i.p. routes), sample S1 was found to show no untoward effect and the degree of infiltration was within the normal range. In saline control (i.p. and i.v. routes) mice, the degree of infiltration was within the



i. ii.

Fig. 3. Photomicrographs showing hematoxylin and eosin (H&E) stained sections of the lungs from mice receiving: (i) only chitosan 2 g/kg, and (ii) saline. A severe form of hemorrhagic, interstitial pneumonia after 48 h of taking the chitosan injection is shown in (i). The alveolar space is reduced drastically, scale bar = 45 μm. Normal histological patterns of the lung tissues from mice injected only with saline, scale bar = 45 μm, is depicted in (ii).

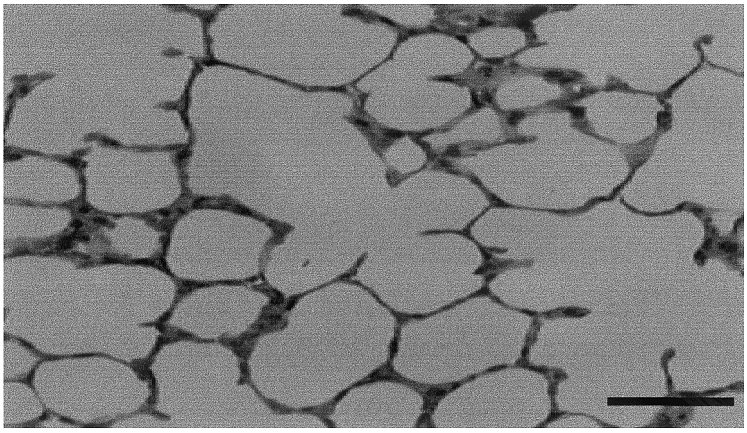


Fig. 4. Photomicrograph showing H&E stained section of the lungs from mice receiving 1 gm/kg intraperitoneal chitosan and simultaneous intravenous P-chitin at the rate of 8 mg/kg (DS of 58%; M_n of 24 000) treatment. There is no infiltration in the interstitial areas of the lung tissue, scale bar = 45 μm. (Photomicrographs of Figs. 3 and 4 were retrieved from low power lens with a magnification of: × 200).

normal value. Only P-chitin (S1) was found to exhibit significant activity irrespective of its i.v. or i.p. route of administration (Table 2).

3.3. Relationship between the amount of P-chitin and chitosan administered, and the degree of infiltration in the lungs (Experiment 2)

Based on the result obtained from Experiment 1, the best P-chitin (S1) was further tested at the rate of 40 mg/kg (i.v.) level in mice challenged by chitosan at the rate of 2 g/kg. Intravenous administration of the P-chitin (S1) solution at the rate of 40 mg/kg (5 mg/ml) was found to block the chitosan challenge successfully, although per area infiltration was slightly higher than that found at the rate of 8 mg/kg (i.v.) level in the previous experiment. Mice receiving only P-chitin (S1) at the rate of 40 mg/kg (0.2 ml, i.v.) or 0.2 ml saline by i.v. route did not develop peritonitis and discernible pneumonic lungs but some degree of interstitial infiltration was evident with the former as shown in Table 3. When the degree of infiltration of P-chitin treatment (S1) in Experiments 1 and 2 were compared, it was comparatively higher in Experiment 2 owing to the severity of lung involvement resulting from the higher amount of chitosan administration.

3.4. Comparison of other P-chitin formulations (S4–S9) for their protective effects against chitosan induced pneumonia (Experiment 3)

Among the six types of P-chitin tested, except for one P-chitin sample (S8 with a DS of 51.5% and M_n of 81 000) all the other samples were found statistically significant ($p < 0.05$) from chitosan control (Table 4). However, for samples S4 and S7, per area infiltration of the lungs was less than 30% and for S5, S6 and S9, it was just or slightly above 30%, whereas for S8 it was similar to chitosan control. Sample S7 with a DS of 34% and M_n of 37 000 was found to perform better than the rest.

4. Discussion

The most significant findings in this investigation were as follows: (1) development of hemorrhagic, interstitial pneumonia resembling ARDS by an overdosage of chitosan

Table 3
Results of the image analysis of the histological lung sections of mice in Experiment 2. (Per area infiltration was estimated by Image Pro® Plus software (Version 3.0 for Power Macintosh© 1996 Media Cybernetics) as described in the experimental methods. P-chitin (S1) was administered at the rate of 40 mg/kg (0.2 ml of 5 mg/ml solution) intravenously. The significance difference between superscript ab, ac, and ad are $p < 0.0001$, $p < 0.01$ and $p < 0.0001$, respectively)

Groups	Number of animals ($n = 20$)	Per area infiltration in the interstitial spaces of the lung (mean ± std. dev.)
I. Chitosan (CTS) control	8	
i. 2 g/kg, i.p.	5	45.79 ± 13.70^{a}
Ii. 4 g/kg, i.p.	3	53.06 ± 7.49
II. CTS 2 g/kg + P-chitin (S1), i.v.	6	27.62 ± 4.83^{b}
III. P-chitin control (S1), i.v.	3	35.23 ± 4.50^{c}
IV. Saline control, i.v. (0.2 ml)	3	27.11 ± 2.69^{d}

Table 4
Results of the image analysis of the histological lung sections of mice for evaluation of six other P-chitin (S4–S9) in Experiment 3. (Per area infiltration was estimated by Image Pro® Plus software (Version 3.0 for Power Macintosh© 1996 Media Cybernetics) as described in the previous experiments. Data of per area infiltration are displayed as mean ± std. dev. for all groups. The level of significance with respect to chitosan control are: ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$. Chitosan control, saline control and P-chitin (S1) from Table 3 are listed for comparison

P-chitin (at the rate of 40 mg/kg, i.v.) (Mn/DS/DDA)[a]	Number of animals ($n = 18$)	Per area infiltration in the interstitial spaces of the lung (mean ± std. dev.)
Chitosan control (2 g/kg), i.p.	5[b]	45.79 ± 13.70
S1(24 KD/58.0/4.0)	6[b]	27.62 ± 4.83****
S4 (22 KD/23.5/7.0)	3	27.79 ± 1.89****
S5 (25 KD/37.5/8.0)	3	30.71 ± 8.65***
S6 (39 KD/58.0/0.0)	3	32.10 ± 9.14**
S7 (37 KD/34.0/3.0)	3	25.80 ± 2.85****
S8 (81 KD/51.5/5.0)	3	44.96 ± 9.73
S9 (78 KD/78.5/9.0)	3	32.12 ± 5.67**
Saline control (0.2 ml), i.v.	3[b]	27.11 ± 2.69****

[a] Median molecular weight (M_n) 1000 = 1 KD; KD: Kilo Dalton.
[b] Mice used in Experiment 2 (listed only for comparison). Other mice received a simultaneous injection of intraperitoneal (i.p.) chitosan at the rate of 2 g/kg, and intravenous (i.v.) P-chitin at the rate of 40 mg/kg (0.2 ml of 5 mg/ml solution).

in mice model, and (2) the blocking effect of certain P-chitin formulations when administered simultaneously with chitosan challenge.

To our knowledge, we are at present the only laboratory investigating the protective effects of P-chitin therapy in a mice model of chitosan-induced ARDS. This ARDS model was similar to the model developed by Takayama (1996). The histological findings of the present model such as an extensive and diffuse interstitial and alveolar infiltration by PMN leukocytes in addition to edema and severe hemorrhage were consistent with shock lung and ARDS as described by Murray (1974); Lesur et al. (1999), respectively. Mice receiving only chitosan and/or P-chitin treatment were showing varying degrees of dullness when compared to mice administered only with saline. Intraperitoneal administration of chitosan alone produced a different degree of reactions ranging from mild pneumonia and peritonitis to severe pneumonia and severe peritonitis in a dose and time-dependent manner. In mice challenged with chitosan when injected with i.v. P-chitin (DS of 58% and M_n of 24 000), pneumonia could be completely blocked. Similarly, when mice challenged with chitosan received the same P-chitin (S1) by i.p. route, the degree of blockage was less effective than that conferred by the i.v. injection, although pneumonia blocking activity was significant when compared to other P-chitin samples ($p < 0.05$). Only one P-chitin (S1) could significantly block pneumonia ($p < 0.05$), irrespective of its routes of administration, whereas P-chitin samples S2 and S3 were not effective by both routes. From our present work, it was found that not all the P-chitin samples were effective in blocking pneumonia. Based on Experiment 1, the DS seems to be an important factor in exhibiting the desired anti-inflammatory activity since the DDA and M_w were almost similar or were in close range among the three P-chitin (S1–S3) formulations. Whereas, after having tested six more samples in Experiment 3, only three formulations (S1, S4 and S7) were found to be effective. From Experiments 1–3, we could not establish as yet which chemical characteristics are important for the exhibition of pneumonia blocking activity. More elaborate in vivo studies using a large number of samples will help to identify the important factors responsible for the protective effects.

The endothelial adhesion and aggregation of PMN leukocytes besides pulmonary hemorrhages and edema seen on a microscopic examination of the chitosan induced pneumonic lungs of mice in our present work may be attributed to the effect of C5a. This finding is consistent with the findings of Minami, Suzuki, Okamoto, Fujinaga and Shigemasa (1998) in dogs. C5a being a very potent chemotactic factor can induce degranulation of mast cells and basophils and provoke the release of histamine, increase capillary permeability and smooth muscle contraction, and even a very small amount (5–10 mg/ml) can provoke direct migration of PMN cells, monocytes and eosinophils (Labadie, 1993). An overdose of chitosan administration in our present work may release many inflammatory stimuli that result in activation and stiffening of the PMN and subsequent sequestration within the microvasculature of the lung and other organs. This finding is in close agreement to that reported by Inano, English and Doerschuk (1992), wherein zymosan activated plasma induced stiffening of PMN and caused a rapid decrease in the deformability of PMN due to the formation of F-actin. Leukocytes accumulated at the sites of inflammation are believed to contribute to tissue damage by releasing lysosomal enzymes and reactive oxygen species (Croft, 1993). While inducing pneumonia by chitosan numerous cells may be stimulated, which result in the synthesis of the prostanoids, leukotriens, and platelet-activating factor (PAF) from precursor fatty acids and phospholipids. The mechanism by which P-chitin blocks chitosan-induced pneumonia is not yet clearly understood at the moment, and is under investigation. However, it is

speculated that P-chitin exerts its mechanism of action either by blocking complement activation or restoring normal surfactant functions or by preventing endothelial adhesion of leukocytes or by restoring the ability of PMN cells to be deformable. Moreover, because of its anionic nature and heparinoid-like activity, it is presumed that P-chitin will not bring about much inflammatory cells, and may also behave like a natural surfactant in already deranged pulmonary surfactant functions due to systemic insult resulting from chitosan. By preventing the migration of PMN cells from the systemic circulation, P-chitin may help to prevent ultimate lung damage by the release of pro-inflammatory mediators from the activated PMN cells and resident alveolar macrophages in the lungs. Ongoing investigations are expected to elucidate the possible mechanism of action.

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