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Phosphated chitin (P-chitin) exerts protective effects by restoring the deformability of polymorphonuclear neutrophil (PMN) cells

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Abstract

In continuation of our on-going investigation on P-chitin, we screened 11 more formulations, out of which only two of them were found effective in vivo. It was not known at that moment, why all the formulations were not effective in vivo. Based on the results of the in vivo screening experiment, in vitro tests were carried out to determine the influence of two effective and non-effective samples on migrating ability of polymorphonuclear neutrophil (PMN) cells. PMN cells were made to loose migrating ability to mimic the in vivo condition of PMN sequestration as it occurs during the acute lung injury, by pre-incubating with chitosan activated serum (CAS). To our surprise, such CAS treated PMN cells with reduced ability to migrate, when subjected to P-chitin treatment were found to have restored the migrating ability. Interestingly, only those samples which were effective in vivo enhanced the deformability or the migrating ability of PMN cells, whereas non-effective samples hindered it. How effective P-chitin restores PMN cells deformability is under investigation.

Keywords: Chitosan activated serum; Polymorphonuclear neutrophil; Migrating ability

1. Introduction

Recently publications addressing the versatilities of chitin and chitosan biomaterials in the biomedical fields are increasing. Usage of chitin and chitosan in veterinary practices has already been documented in detail (Minami, Okamoto, Matsuhashi, Sashiwa, Saimoto, Shigemasa et al., 1992; Okamoto, Minami, Matsuhashi, Sashiwa, Saimoto, Shigemasa et al., 1992; Minami, Okamoto, Hamada, Fukumoto & Shigemasa, 1999). Earlier, we reported on the protective nature of some P-chitin formulations (Khanal, Miyatake, Okamoto, Shinobu, Shigemasa, Tokura et al., 2001) and in this investigation, additional screening tests were performed with 11 more formulations to find out the relationship between the chemical characteristics and the protective effect, if any. Besides, in vitro migration test was carried with the view of unveiling the possible mechanism(s) of action.

Since the reasons for varying levels of anti-inflammatory activity of P-chitin in vivo are not completely understood at the moment, the present in vitro study was aimed at seeking the probable reasons for such in vivo variations besides elucidating the mechanism of action, if any. In patients with acute respiratory distress syndrome (ARDS), C5a has been reported in the plasma with the evidence of early neutropenia subsequent to intravascular leukoagglutination and sequestration of aggregated cells within the pulmonary vascular beds that would result in the production of toxic oxygen metabolites detrimental to lung parenchyma (Hammerschmidt, Weaver, Hudson, Craddock & Jacob, 1980; Till, Johnson, Kunkel & Ward, 1982). The work by Inano, English and Doerschuk (1992) has revealed that zymosan-activated plasma induced stiffening of polymorphonuclear neutrophil (PMN) and subsequent loss of deformability. Similar to zymosan-activated plasma induced stiffening of PMN, chitosan activated serum (CAS) also results in PMN (CAS-PMN) stiffening with subsequent loss of deformability and hence, we used CAS for rendering PMN cells to loose deformability for mimicking the in vivo condition of PMN sequestration in the lungs parenchyma, as it usually occurs in the early course of acute respiratory distress syndrome. There is an increasing body of speculation on the new candidate drugs for being effective,

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Table 1
Results of histological evaluation of 11 formulations of P-chitin (S10–S20). (M_w (average molecular weight): GPC analysis; KD: Kilo Dalton; DS (degree of substitution): elemental and ^{1}H NMR analyses; DDA (degree of deacetylation): ^{1}H NMR analysis; Initial pH of P-chitin (1 mg/ml) solution was measured at 25°C; (+): gain in body weight, (−): loss in body weight; Data on per area infiltration are displayed as mean ± standard deviation for all groups; The level of significance (unpaired *t*-test, MS Excel, 2000) with respect to chitosan control are: $^{**}p < 0.01$; $^{*}p < 0.05$; E: effective; PE: partially effective and NE: not effective; Data for chitosan and saline control are listed only for comparison)

P-chitin (8 mg/kg, i.v.) (M_w/DS/DDA/pH)	Number of animals ($n = 33$)	(%) Body weight loss or gain (24 h)	Per area infiltration[a] (mean ± SD)
Chitosan control (2 g/kg), i.p.	5	17.26 (−)	45.79 ± 13.70
S10 (52 KD/11.0/3.0/6.99)	3	8.00 (−)	27.67 ± 2.80** E
S11 (50 KD/18.5/0.0/6.30)	3	16.88 (−)	30.94 ± 3.19** PE
S12 (50 KD/47.5/4.0/6.93)	3	15.58 (−)	36.63 ± 7.97 NE
S13 (42 KD/55.0/15.0/6.43)	3	13.75 (−)	48.92 ± 20.72 NE
S14 (80 KD/26.5/3.0/6.65)	3	2 dead	47.67 ± 18.48 NE
S15 (160 KD/41.5/2.0/7.40)	3	12.98 (−)	55.22 ± 14.55 NE
S16 (270 KD/59.0/12.0/7.67)	3	11.84 (−)	42.61 ± 7.07 NE
S17 (53 KD/56.5/8.0/7.15)	3	18.87 (−)	31.09 ± 2.20** PE
S18 (49 KD/53.0/15.0/6.34)	3	14.33 (−)	32.67 ± 5.84* PE
S19 (71 KD/71.0/5.0/6.57)	3	10.57 (−)	32.03 ± 5.21* PE
S20 (130 KD/72.0/3.0/6.87)	3	1.32 (+)	26.23 ± 1.30** E
Saline control (0.2 ml i.v.)	3	1.96 (+)	27.11 ± 2.69**

[a] Per area infiltration in the interstitial spaces of the lung was estimated by Image Pro® Plus software (Version 3.0 for Power Macintosh, ©1996 Media Cybernetics).

provided if they could correct microtubule defect and thereby restore PMN deformability. In our present study, we have mainly attempted to determine the influence of effective and ineffective P-chitin treatment on the restoration of deformability of CAS-PMN cells. We particularly quantified the migrating ability of PMN cells against chemoattractant placed in the lower wells of the chemotaxis chambers after suitable treatment with effective or non-effective P-chitin samples.

2. Experimental

2.1. Preparation of chemicals and reagents

2.1.1. Chitosan and phosphated chitin (P-chitin) samples

Chitosan sample for the development of pneumonic model was a kind of gift from Sunfive Inc., Japan. In brief, chitosan powder synthesized from snow crab shells chitin (Sunfive Inc., Japan), having >82% deacetylation (DDA) and particles <10 μm (mean particle size: 5 μm) was used in this study. The distribution of granule size was measured with 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). Chitosan powder was sterilized by ethylene oxide gas, and was suspended in sterile physiological saline before use. The chitosan suspension was prepared aseptically at a concentration of 100 mg/ml and stored in the refrigerator.

P-chitin samples (11 types) for the in vivo and in vitro investigations were synthesized using phophorylation method as described by Tokura and Tamura (1998). Briefly, 30 g urea was suspended in 30 ml of *N*,*N*-dimethyl formamide (DMF) with stirring until it became a homogenous solution at 100°C, and into which was added 2 g of dried, fine chitin powder from squid pen till the added chitin was swollen. After 5.2 ml of *ortho*-phosphoric acid was added, the reaction mixture 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 disappeared and became colorless. The residue was dissolved in water and the 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 separated was treated by acetone followed by methanol rinse and air-drying. All samples were characterized by the same method as described for earlier samples S1–S9 (Khanal et al., 2001). The chemical characteristics, and the initial pH profiles of all 11 P-chitin samples (S11–S20) are listed in Table 1.

2.1.2. Hank's balanced salt solution (HBSS) buffer

HBSS buffer was prepared by dissolving 9.8 g powdered Hank's balanced salt solution (Hank's Solution 'Nissui'® without phenol red, Nissui Pharmaceutical Co., Ltd, Japan) in distilled water and the volume was adjusted to 1000 ml. After complete dissolution, pH was adjusted (7.3–7.6 at 37°C) by adding a proper amount of sodium bicarbonate and finally sterilized by 0.45 μm filter (Millipore Corporation). It was stored in the refrigerator before use.

2.1.3. Ficoll–Conray solution (Sp. gr. 1.083)

To 130 ml double distilled water, 11.7 g Ficoll powder (Ficoll® 400, Lot No. 226414 Pharmacia Biotech, Sweden) was added and gently heated with stirring. The specific gravity was less than 1.06. This solution was sterilized by autoclaving at 121°C with 15 pounds pressure for 15 min. To the above Ficoll solution, sterile Conray® 400 solution (66.8% w/v sodium iotalamate, Dai-Ichi Seiyaku® Corp., Japan) was mixed at 63:10 proportions, respectively, to achieve a final sp. gr. of 1.083–1.085 at 15°C. The resultant Ficoll–Conray mixture was stored at 2–8°C until its use.

2.1.4. Lysing solution

Lysing solution was prepared as described by Roos and de Boer (1986). To 100 ml double distilled water, 830 mg ammonium chloride (155 mM NH_4Cl), 100 mg potassium bicarbonate (10 mM $KHCO_3$) and 4.0 mg EDTA (0.1 mM EDTA 2K) were dissolved, pH was adjusted to 7.4 at 0°C and was finally sterilized by 0.45 μm filter (Millipore Corp., USA).

2.2. Animal

Five to six weeks old ($n = 33$), female ddy mice weighing approximately 25 g (20–30 g) were used for in vivo study after one week of acclimatization at the laboratory environment. They were fed with commercial chow and drinking water ad lib. Eleven formulations of P-chitin having different pH, degrees of substitution (DS), degree of deacetylation (DDA), and molecular weight (M_w and M_n) were screened in 33 mice. Four 2–5 years old male healthy Beagle dogs weighing 9–15 kg were used for collecting PMN cells to study neutrophilic chemotaxis.

2.3. Administration of P-chitin

We administered 11 formulations of P-chitin (8 mg/kg) by single intravenous (i.v.) injection in mice ($n = 3$ for each P-chitin sample) challenged with chitosan (2 g/kg, i.p.) as described previously, and observed for 48 h. Determination of protective effect of P-chitin against chitosan-induced pneumonia was performed by histological evaluation of lung sections with computer aided image analysis. The degree of infiltration was found to be less than 30% in normal or mice receiving saline or effective P-chitin samples and the same criterion was also used in this experiment as before. Other protocols for the entire experiment were similar to the earlier investigation (Khanal et al., 2001).

2.4. Study on migrating ability of PMN cells

2.4.1. Isolation of PMN cells

For isolation and purification of PMN cells from four healthy dogs, blood was collected in a vacutainer (Venoject, Terumo™, Japan) containing EDTA as an anticoagulant. PMN cells were isolated by density gradient centrifugation as described by Roos and de Boer (1986) with some modification (Usami, Okamoto, Minami, Matsuhashi, Kumazawa, Tanioka et al., 1994). Briefly, 2 ml blood was collected from the jugular vein in a vial containing EDTA as an anticoagulant. A small amount (2 ml) of blood was diluted with 4 ml of HBSS buffer, layered over 6 ml of Ficoll–Conray solution (sp. gr. 1.083) and centrifuged at 2100 rpm for 21–25 min at 4°C to obtain PMN cell rich suspension. The pellet was treated with chilled lysing solution (0.83% ammonium chloride solution containing 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.4 at 0°C) for 7–10 min in ice-bath with 3–4 gentle mixings to hemolyze red blood cells (RBC). Upon complete hemolysis, which was characterized by cherry red color, the tube was centrifuged at 1100–1300 rpm, 4°C for 5 min. The cell suspension was then washed 2–3 times with HBSS buffer by centrifuging at 600 rpm for 3–5 min in room temperature. PMN cells were made free from RBC contamination and were finally suspended in HBSS buffer at a concentration of approximately 2×10^5 cells/ml. Purity and viability (trypan blue dye exclusion test) of the PMN cells were above 90 and 94%, respectively.

2.4.2. Chitosan-activated serum (CAS)

CAS was prepared by incubating 20 mg of chitosan suspension (100 mg/ml) in 1 ml auto-logous canine serum at 37°C for 15 min followed by centrifugation (2500–3000 rpm). Upper supernatant layer was collected and diluted with HBSS buffer to make 10% diluted CAS solution.

2.4.3. Heat inactivated chitosan-activated serum (CAS56)

CAS56 was prepared by incubating 10 mg of chitosan suspension (100 mg/ml) in 0.5 ml auto-logous canine serum at 56°C for 30 min, followed by centrifugation (2500–3000 rpm, 10 min). Upon centrifugation, upper supernatant layer was collected and diluted with HBSS buffer to make 10% CAS56 solution.

2.4.4. CAS treated PMN (CAS-PMN) and P-chitin treated CAS-PMN

Specified amount of PMN cells (3 ml here, but can be varied as per the experimental design) were pre-incubated with CAS (500 μl CAS) at 37°C for 10 min and then washed with 5 ml HBSS buffer by gentle centrifugation (600–700 rpm) and aspiration. The volume of CAS-PMN maintained after washing was 3 ml. Such CAS-PMN cells were then subsequently treated with two effective and two non-effective P-chitin (P-chitin treated CAS-PMN) samples. To every 450 μl CAS-PMN, 150 μl P-chitin (1 mg/ml) was added and pre-incubated at 37°C for 10 min followed by washing with HBSS buffer and gentle centrifugation (600–700 rpm). CAS56-PMN and P-chitin treated CAS56-PMN were also prepared in the similar way.

2.4.5. Measurement of neutrophilic chemotaxis

Quantitative measurement of the migrating ability or

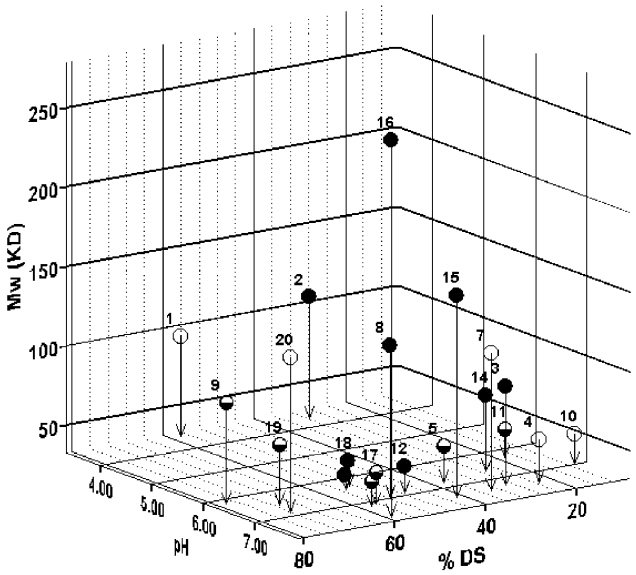

Fig. 1. Showing the relationship between the protective effects and average molecular weight (M_w), degree of substitution (%DS) and pH of P-chitin formulations. Scatter plot made by plotting the independent variables against each other for all 20 samples. All formulations were assigned with their respective numbers just above the symbol (Samples 6 and 13 are about to merge with samples 17 and 18, respectively). Effective samples were chosen on the basis of reduced degree of infiltration in the lung sections after their administration against chitosan challenge (S10–S20 in our present investigation and S1–S9 in the previous work, Khanal et al., 2001). Degree of infiltration was found less than 30% in the lungs of normal mice receiving physiological saline and hence this figure was chosen for grading the samples as effective or ineffective. Effective samples (S1, S20, S10, S4 and S7 marked by empty circles) and some partially effective samples (S9, S11 and S19 marked by half-filled circles) lie at the two extremities, while most ineffective samples (completely filled circles) and few partially effective (S6 and S17) lie in the central zone.

deformability of isolated PMN cells was performed with established protocols (Latimer, Crane & Prasse, 1980; Usami et al., 1994) using non-disposable chamber (Neuro Probe Inc., USA) along with isopore track-etched polycarbonate membrane filters. As a chemotactic substance, 200 μl of 10% diluted, auto-logous serum was added in the lower wells. Polycarbonate filters (Isopore™ Membrane Filters, Cat. No. TMTP01300, Millipore, Ireland) with a pore size of 5 μm were placed between the lower and upper wells. In the upper wells, 200 μl of PMN cells after pre-incubation with or without CAS followed by P-chitin or without P-chitin treatment was added. PMN cells treated differently were allowed to migrate towards the chemotactic substance placed in the lower wells of the Boyden chambers. Random migration was determined by placing 200 μl HBSS buffer instead of serum in the lower wells. All tests were run into duplicates and repeated at least three times. Boyden chambers were incubated for 45 min at 37°C in humidified CO_2 incubator. After incubation, upper chamber's fluid content aspirated, filter was removed, inverted, air-dried and fixed in methanol. All the filters were then stained by Diff-Quick (International Reagents Corporation, Japan) stain, and after drying were examined under 40X (400 magnification) of the microscope. PMN cells migrated completely across the membrane was quantified by identifying and counting the number of cells that were in filter visually in a specified area (visual end point assay). Twenty fields were randomly selected and number of PMN migrated to the lower surface of the filters were counted and averaged. PMN cells thus migrated were compared to that of normal PMN cells or those pre-incubated with CAS (CAS-PMN) or P-chitin treated CAS-PMN. Results were expressed in terms of the percentage of PMN cells migrated to the lower surface of the filter.

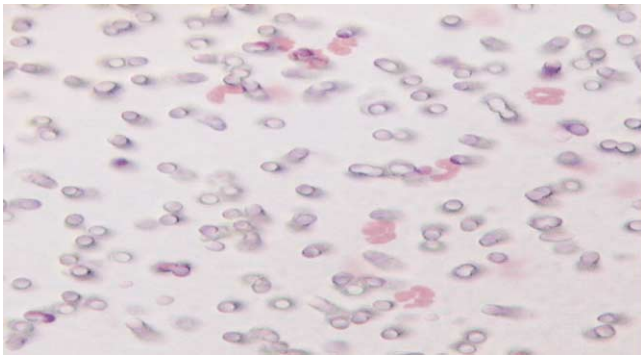

Fig. 3. Showing the migration profiles of CAS-PMN cells towards the chemoattractant placed in the lower well of the chamber (× 400). Note that very few neutrophils migrated to the lower surface of the poly-carbonate filter (5 μm pore size).

3. Results

3.1. Evaluation of different P-chitin formulations (S10–S20) for their protective effects

Based on the computer aided image analysis, among the 11 formulations of P-chitin (S10–S20) screened, only two samples (S10 and S20) were found effective in preventing chitosan-induced pneumonia (Table 1). The degree of infiltration in the histologic lung sections was less than 30% for the effective samples, while for some other samples, it was just around borderline or little above the border line and hence were named as partially effective (S11, S17–S19).

Apparently, no linear relationship was observed between the individual parameters like DS, DDA, M_w and pH of P-chitin samples with the degree of infiltration in the lung sections. However, from multiple regression analysis, the chemical parameters M_w, DS and the initial pH of P-chitin seemed to have profound influence on the efficacy of P-chitin samples for minimizing the degree infiltration in the lungs. From the three dimensional scatter-plot diagram (Fig. 1) of the chemical parameters, M_w, DS and pH of all P-chitin samples (present 11 and previous nine samples) in relation to the degree of infiltration, it was found that samples having either low molecular weight and low %DS (S4 and S10), or

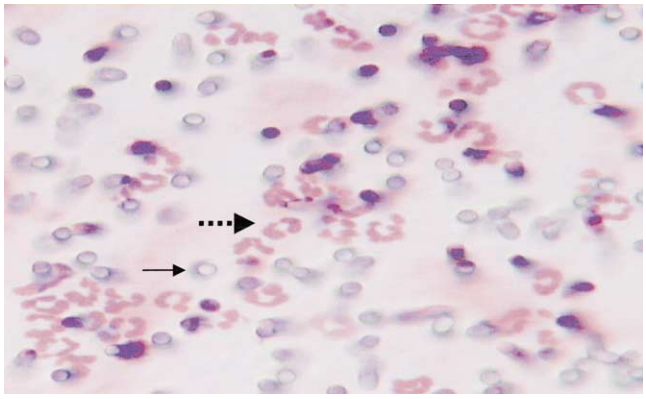

Fig. 2. Showing the migration profiles of normal PMN cells towards the chemoattractant placed in the lower well of the chamber (× 400). Note that many neutrophils migrated to the lower surface of the polycarbonate filter (small solid arrow: 5 μm pore size; large dotted arrow showing migrated neutrophilis).

high molecular weight with slight to moderately high %DS (S1, S7 and S20) were effective in conferring protection against chitosan-induced pneumonia.

3.2. Migrating ability PMN cells after P-chitin treatment

From the Boyden chamber experiments, it was found that PMN cells treated with CAS (CAS-PMN) lost migrating ability (Fig. 3) significantly ($p < 0.01$) when compared to normal PMN cells (Fig. 2) whereas, when such CAS-PMN cells were allowed to migrate after P-chitin treatment, restored their deformability (Fig. 4). Among four formulations tested, only those samples that were effective in vivo were found to restore PMN deformability significantly (Fig. 5) while ineffective samples were not. Likewise, PMN cells pre-incubated with heat inactivated CAS (CAS56) also lost migrating ability but not to the extent of CAS-PMN, and

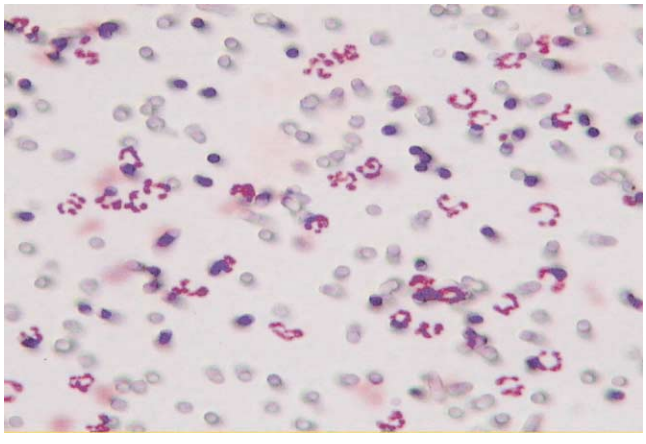

Fig. 4. Showing the migration profiles of P-chitin treated CAS-PMN cells towards the chemoattractant placed in the lower well of the chamber.(× 400). Note that many neutrophils have restored the migrating ability to the lower surface of the poly-carbonate filter (5 μm pore size).

were found to regain almost full migrating ability after treatment with effective P-chitin sample.

4. Discussion

The main findings in this investigation were as follows: (1) superiority of either low molecular weight P-chitin sample with low DS (S10) or high molecular weigh P-chitin sample with high DS (S20) for conferring protection against chitosan-induced pneumonia, (2) inhibition of the migrating ability or deformability of PMN cells after pre-incubation with CAS and, (3) the subsequent restoration of such PMN cells' deformability by effective P-chitin treatment.

From our present work, it was found that not all P-chitin samples were effective in protecting the mice against chitosan-induced pneumonia. Among the 11 P-chitin samples tested, it was found that samples having either low M_w and low DS (S10 and S11 were partially effective) or high M_w and higher DS (S20) effective in protecting the lungs against chitosan challenge. Other samples with low M_w and high DS or high M_w and low DS were not effective. There were also varying degrees of weight loss in mice receiving chitosan and different formulations of P-chitin, less severe loss with effective samples than with chitosan control or treatment with ineffective P-chitin samples.

With the in vitro migration study of PMN cells using four formulations of P-chitin, it was found that only those samples which were effective in vivo restored PMN cells' deformability significantly, while ineffective samples did not. Besides, pre-incubation of PMN cells with heat inactivated CAS (CAS56-PMN) did not reduce the migrating ability to the extent of CAS-PMN but there was some degree of reduction when compared to that of normal PMN. CAS56-PMN cells when treated with effective P-chitin have almost restored their full migrating ability. This finding is suggestive of the important role of thermo labile components of the complements such as C3, for activation of the PMN cells and their subsequent loss of deformability. It is implicated that both C5a and C3a are generated from activated complements in CAS and resulted in the decreased deformability of PMN cells, whereas in case of CAS56, only thermo-stable component such as C5a gets activated and it has less profound effect than the additive effect of C5a and C3a on the deformability of PMN cells. Minami, Masuda, Suzuki, Okamoto, Matsuhashi, Kato et al., 1997 have reported about the significant rise in C3 level and activation of PMN cells after chitosan administration in dogs. Complements components C3 and C5, but not C4 were activated by chitin and chitosan via the alternative pathway especially with greater intensity from chitosan (Minami, Suzuki, Okamoto, Fujinaga & Shigemasa, 1998). The component C3 is a major plasma glyco-protein, and it plays a central role in the system, being common to both classical and alternative pathways (Law & Reid, 1995). The present study indicated that both heat resistant and heat labile

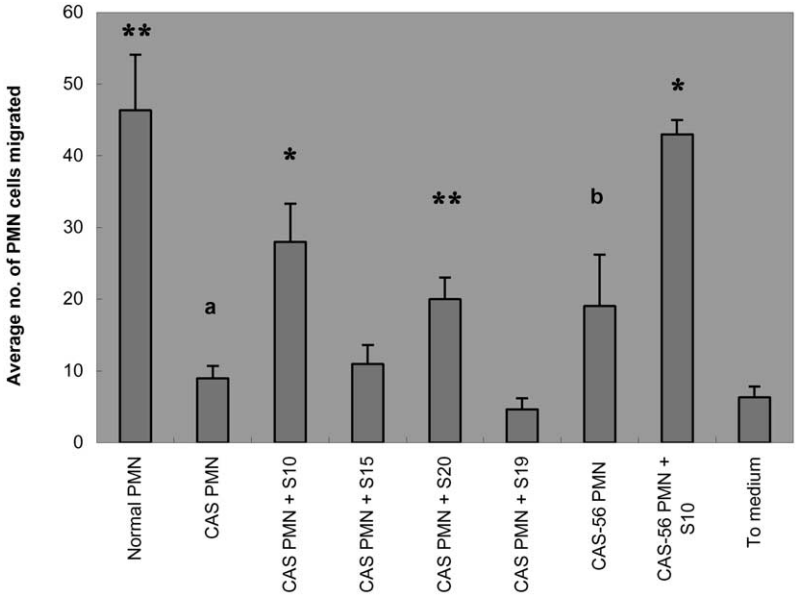

Fig. 5. Showing the average number of PMN cells migrated (mean ± SD) against the chemoattractant (auto-logus serum) after various treatment. Random migration was measured by placing the HBSS medium instead of the chemotactic substance in the lower wells. CAS-PMN (marked as letter 'a') cells treated by P-chitin S10 (CAS-PMN + S10) and S20 (CAS-PMN + S20) were having significantly higher ($^{*}p < 0.05$ or $^{**}p < 0.01$) number of PMN cells' deformability than the CAS-PMN alone or CAS-PMN treated by S15 (CAS-PMN + S15) and S19 (CAS-PMN + S19). PMN cells pre-incubated with heat-inactivated CAS (CAS56-PMN) had slightly reduced migrating ability when compared to the normal PMN but not to the extent of CAS-PMN, but when such PMN cells were treated by P-chitin, S10 restored almost complete migrating ability ($p < 0.05$). Statistical significance ($p < 0.05$) for normal PMN or CAS-PMN + P-chitin were tested against CAS-PMN (marked by letter 'a') and for CAS56-PMN + S10 was against CAS56-PMN (marked by letter 'b').

components such as C5a and C3a played crucial roles on migrating ability of PMN cells especially with more strong effect of heat-labile component, C3a. Since the restoration with effective P-chitin treatment is not complete as compared to that of normal PMN, there may be other mechanisms in addition, which act in a concerted manner to exert protective effects in vivo.

An over dose of chitosan challenge may release many inflammatory stimuli that result in activation and stiffening of the PMN cells and subsequent sequestration within the micro-vasculature of the lungs and other organs. Our present study with CAS-PMN is an attempt to simulate the in vivo condition of stiffening PMN cells by chitosan over dosing. The present findings of reduced deformability of PMN cells after pre-incubation with CAS is in close agreement to that reported by Inano et al. (1992) wherein zymosan activated plasma induced stiffening of PMN, and caused a rapid decrease in the deformability of PMN. We also found reduced deformability of PMN cells (data not shown) after pre-treatment with zymosan-activated serum (ZAS). It is speculated that the same situation may prevail inside the mammalian body and leukocytes thus accumulated at the sites of inflammation result in tissue damage by releasing lysosomal enzymes and reactive oxygen species (Croft, 1993). The mechanism how P-chitin helps in the restoration of PMN cells' deformability is partially uncovered. There have been some reports on defective cyclic nucleotide metabolism within the phagocytes such as PMN cells, which results in poor micro-tubule function and defective chemotaxis (Harvath, Robbins, Russell & Seamon, 1991). Hatch, Nichols and Hill, 1977 reported that cAMP depresses neutrophil migration towards chemoattractant whereas cGMP enhances the same process. Levamisole also significantly enhanced chemotaxis of PMN cells (Hogan & Hill, 1978). Preliminary data on cyclic nucleotides have revealed that CAS treatment resulted in increasing the intracellular cAMP levels whereas effective P-chitin treatment did not. This finding is in agreement with what we have speculated that chitosan causes an intracellular elevation of cAMP in PMN cells both in vivo and in vitro, and thereby loose the chemotactic ability and results in pulmonary leukostasis. While P-chitin reverses the stiffening of PMN cells in the tissues and thereby prevents the release of inflammatory mediators. Besides, P-chitin possesses scavenging activity on reactive oxygen metabolites (unpublished report). The mechanism(s) how P-chitin blocks chitosan-induced pneumonia is partially understood at the moment, and more than one mechanism of actions seem to act in a concerted manner. On-going investigations may unravel the other possible mechanism(s) of action in near future.

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