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Influence of chain length of *N*-acetyl-D-glucosamine and D-glucosamine residues on direct and complement-mediated chemotactic activities for canine polymorphonuclear cells

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The direct and complement-mediated chemotactic activities (CA) of *N*-acetyl-D-glucosamine (GlcNAc), D-glucosamine (GlcN), GlcNAc and GlcN oligomers, chitin and chitosan for canine polymorphonuclear cells (PMNs) were investigated *in vitro*. GlcNAc6 and GlcN5~6 provided the most effective stimulation of CA, with GlcN6 having a seven-fold stronger effect than GlcNAc6. Serum incubated with chitin and chitosan (37°C, 30min) had a similar CA to that of zymosan. The total complement activity (CH50) of serum mixed with chitin and chitosan was lower than control level, but monomers and oligomers of GlcNAc or GlcN did not decrease complement activity. Subcutaneous injection of GlcN6 and chitosan enhanced more PMN migration than GlcNAc6 and chitin *in vivo*. It was demonstrated that GlcN residues induced higher direct CA than GlcNAc residues, while complement-mediated CA was only induced by chitin and chitosan, but not by GlcNAc, GlcN and their oligomers. © 1997 Elsevier Science Ltd

INTRODUCTION

Chitin and chitosan, polysaccharides made up of β-(1→4)-linked GlcNAc and GlcN units, are widely found in nature, forming skeletons of crustaceans and insects as well as components of bacterial cell walls. We have demonstrated that chitin and chitosan are useful in veterinary practice as wound healing accelerators (Minami *et al.*, 1992, 1993; Okamoto *et al.*, 1992, 1993b). However, the mechanism of the improvement of wound healing by these agents has not been completely examined. We previously reported that chitin and chitosan were chemoattractants for canine and bovine PMNs, which play an important role in wound healing (Usami *et al.*, 1994a, b). In addition, we investigated whether chitin and chitosan were completely biodegradable in animal subcutaneous tissues (Okamoto *et al.*, 1993a). However, it has remained unclear whether oligomers and monomers of GlcNAc and GlcN, which would be produced during degradation of chitin and chitosan, act as PMNs chemoattractants or not. When chitin and chitosan are applied to a wound, they initially come into contact with exudate or blood. It is well-known that wound exudate has a high content of complement, which is also a strong PMN chemoattractant (Snyderman *et al.*, 1971). However, few studies have investigated complement activation by chitin and chitosan.

Against this background, the present study investigated the effect of the chain length of GlcNAc and GlcN residues on stimulation of canine PMN migration and on serum complement levels.

EXPERIMENTAL

Materials

Chitin and chitosan.
Pulverized chitin powder (Sunfive, Japan), purified from loligopen and chitosan powder and chemically deacetylated from snow crab shell chitin (Sunfive,

Japan), was suspended in Hank's balanced salt solution (HBSS, Nissui Seiyaku, Japan) for use in this study. The chitin and chitosan used showed $<10\%$ and $>80\%$ deacetylation, respectively. The mean particle size of each powder was 3.5μm, as measured with an SK Laser Micron Sizer 7000S (Seisin K. K., Japan).

Oligomers and monomers of GlcNAc and GlcN.
Oligomers and monomers of GlcNAc and GlcN were obtained by depolymerization with partial acid hydrolysis of chitin and chitosan prepared from snow crab shell. Mixtures of GlcNAc1~GlcNAc6 (MNG) and GlcN1~GlcN6 (MG), as well as chromatographically homogeneous GlcNAc1~6 and GlcN1~8, were used in this study (Yaizu Suisankagaku Industries, Japan). Oligomers with an average polymerization of GlcN20 (GlcN2~100 [GlcN20 $<70\%\pm10\%$]) and GlcN35 (GlcN30~40) were also used (Pias, Japan).

Blood collection and animals

Peripheral blood was collected from 10 mongrel dogs aged 1–4 years old (six males and four females), and three mongrel dogs aged 4 years old (three females) were used for PMN migration assay *in vivo*.

Separation of PMNs

Separation of canine PMNs from collected blood was carried out by density gradient centrifugation (S.G.: 1.082), as reported previously (Usami *et al.*, 1994b).

Preparation of serum

Chitin suspended in fresh autologous serum (FSNG) and chitosan suspended in fresh serum (FSG).
Ten mg of chitin or chitosan was suspended in 1ml of fresh autologous serum. Each mixture was diluted to 10% with HBSS and was used rapidly for the chemoattraction studies.

Zymosan-activated serum (ZAS).
Zymosan A (Sigma, USA) was suspended in fresh canine serum (10mg/ml), and the suspension was incubated at 37°C for 30min. Then the zymosan particles were removed by centrifugation at 700×*g* for 10min, the supernatant was heated at 56°C for 30min and the heated serum was diluted to 10% with HBSS, for use as a chemoattractant. Fresh canine serum (FS) and heated-inactivated canine serum (HIS) (56°C for 30min) were prepared by standard procedures and diluted to 10% with HBSS.

Chitin-activated serum (NGAS) and chitosan-activated serum (GAS).
Chitin- and chitosan-activated serum were prepared in the same manner as ZAS.

Measurement of canine PMN migration *in vitro*

The migration of canine PMNs in response to chitin, chitosan, GlcNAc1~6, GlcN1~8, GlcN20, GlcN35, ZAS, NGAS, GAS, FS, HIS, FSNG, and FSG was measured using the blind well chamber (Nucle Pore, USA) method.

Two hundred μl of each sample was placed into the lower chamber. A 5μm pore-sized polycarbonate filter (Neuro Probe, USA) was placed between the lower chamber and the upper chamber. Two hundred μl of cell suspension was added to the upper chamber. The chambers were incubated at 37°C for 60min. Each filter was then removed, air-dried, fixed in methanol, stained with Light-Giemsa solution and mounted on glass slides. The migrated cells which completely passed through the pores and attached to the lower surface of the filter were counted for 30 randomly selected oil immersion fields under a microscope at a magnification of 1000. The result was expressed as the number of PMNs per mm^2 filter surface.

Measurement of CH50

The total complement activity contained in pulverized chitin or chitosan, oligomers or monomers of GlcNAc and GlcN and zymosan, was measured with a complement activity (CH50) determination kit (Denka Seiken, Japan).

In brief, 1ml of fresh serum was mixed with 2, 5 and 10mg of chitin, chitosan, or oligomers and monomers of GlcNAc and GlcN. Each mixture was incubated in a CO_2 incubator (37°C, 30min) and was centrifugated at 700×*g* for 10min, after a while 10μl of the supernatant was added to buffer. Then the buffer was mixed with 50μl of sensitized sheep erythrocyte suspension (4×10^9cells/ml) and incubated at 37°C for 60min. Next, the mixture was placed into an ice-bath for 5min to stop the reaction, and was centrifugated at 700×*g* for 10min. Absorbance of the supernatants was measured at 541nm using a spectrophotometer. After comparison with a water blank, the absorbance values were changed into CH50 values using a conversion table.

Observation of canine PMN migration *in vivo*

Two ml of a 100μg/ml of GlcNAc, GlcN, GlcNAc6 GlcN6, chitin and chitosan in saline (GlcNAc group, GlcN group, GicNAc6 group, GlcN6 group, chitin group and chitosan group, respectively) and 2ml of saline (control group) were subcutaneously administrated to the dogs on both sides of the dorsal midline. Six hours later the dogs were anesthetized with sodium pentobarbital (25mg/kg i.v.), after atropine sulfate (0.05mg/kg s.c.) premedication. A full thickness of skin and subcutis (1×2cm) was surgically removed in all administrated sites. So, three tissues were collected in each group. The removed skin and subcutis were fixed in 10% neutral folmaldehide solution and were embed-

ded in the wax. Thin sections were made by microtome and then a section was stained with hematoxylin and eosin solution after deparaffin. The stained section was observed under a light microscope. The degree of histological findings were rated on a scale of 0–3 as follows: degree of PMN migration, 0 = < 10, 1 = < 100, 2 = < 500, 3 = > 500; which were the average number of migrated PMNs of a field (triplicate) under ×400 magnification. The degree of perivascular infiltration, edema and haemorrhage: 0 = no change; 1 = slight; 2 = moderate; 3 = severe.

Statistical analysis

Statistical analysis was performed with the unpaired student's *t*-test.

RESULTS

The effects of GlcNAc1~6, GlcN1~8, GlcN20 and GlcN35 on the chemotactic activity (CA) of canine PMNs are shown in Figs 1 and 2. There was no significant difference between the CA of GlcNAc1~5 and HBSS, while the CA of GlcNAc6 was significantly higher than that of HBSS. All of the oligomers and monomers of GlcN had a higher CA than HBSS (negative control), and the highest CA was obtained with GlcN5 and GlcN6. The number of PMNs migrating to GlcN or GlcN35 was about one-fourth of the number for GlcN5 and GlcN6. Although the hexamers of GlcN and GlcNAc were the most effective PMN chemoattractants in this assay, the CA of GlcN6 was about seven times higher than that of GlcNAc6.

As shown in Fig. 3, canine PMNs responded additively better to FSNG and FSG than to chitin and chitosan, respectively. Although the number of cells migrating to chitin was 1.5 times greater than that for chitosan, there was no significant difference between FSNG and FSG. Thus, the CA of chitosan was more sensitive to the effect of serum than chitin. Fig. 4 shows the CA of NGAS and GAS. Both NGAS and GAS had CA as high as that of ZAS, and four times higher than that of HIS.

The consumption of total complement by chitin, chitosan, MNG, MG, GlcNAc and GlcN was also evaluated (Figs 5 and 6). Chitin and chitosan consumed more complement than the control, but monomers and oligomers of GlcNAc or GlcN did not consume the complement.

Histological findings of the recovered tissue in each group are summarized in Table 1 and Fig. 7. Aggressive PMNs migration and perivascular infiltration of PMN were observed in GlcN, GlcN6, chitin and chitosan groups than in saline and GlcNAc groups. Especially in chitin and chitosan groups, numerous PMNs migrated to chitin and chitosan particles. Haemorrhage and edema in subcutaneous tissue were more apparent in chitosan and GlcN6 groups than in chitin and GlcN groups.

DISCUSSION

In this study, we demonstrated that some oligomers of GlcNAc and GlcN enhanced CA for canine PMNs.

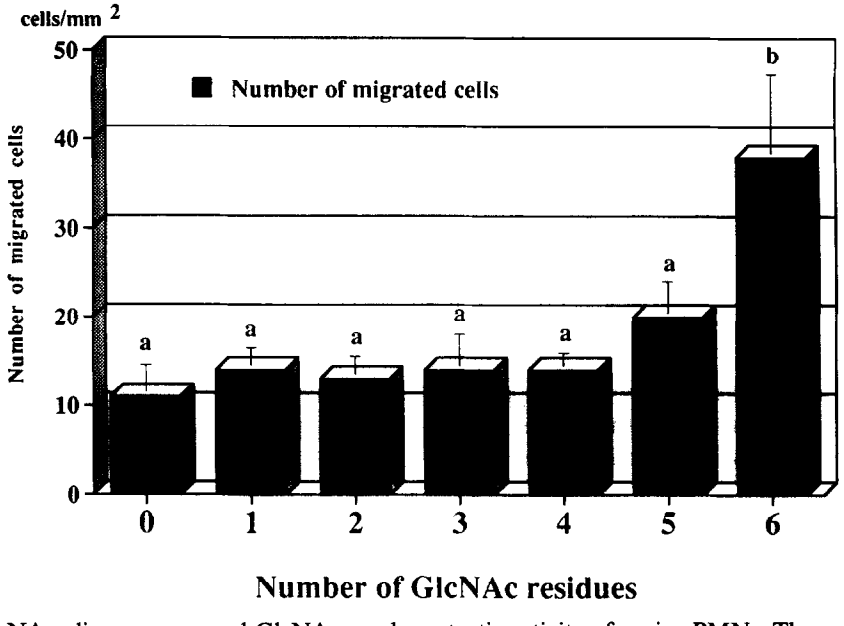

Fig. 1. The effect of GlcNAc oligomomers; and GlcNAc on chemotactic activity of canine PMNs. The concentration of oligomers and GlcNAc was 10^2 μg/ml. No. of GlcNAc residues: 0, HBSS; 1, GlcNAc oligomer of (GlcNAc)*n* (*n* = 2~6). Data were shown as mean±s.d. of duplicate determination for three experiments. The different letters in the figure mean significant difference between them ($p < 0.05$).

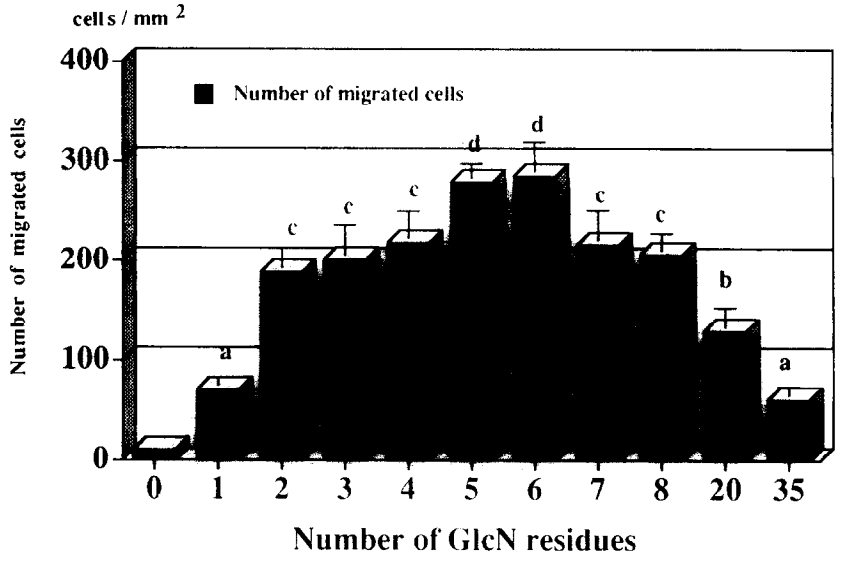

Fig. 2. The effect of GlcN oligomomers and GlcN on chemotactic activity of canine PMNs. The concentration of oligomers and GlcN was $10^2\mu$g/ml. Data were shown as mean±s.d. of duplicate determination for three experiments. No. of GlcN residues: 0 = HBSS, 1–8; oligomer of (GlcN)*n* (*n* = 1~8), 20 = M.W. 400~20,000 (4000 < 70±10%), 35 = M.W. 7000±1500. Each letter in the figure is significantly different from d: a, ($p < 0.001$); b, ($p < 0.01$); c, ($p < 0.05$).

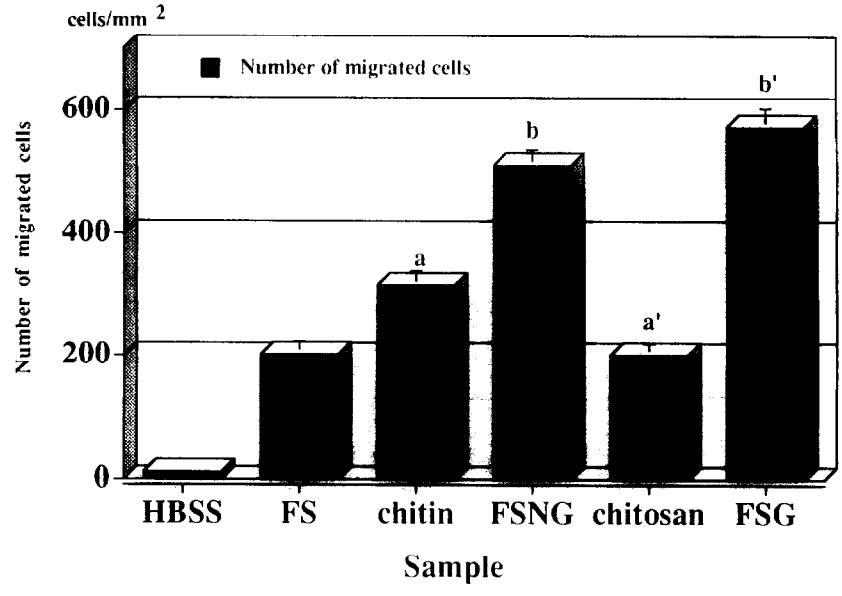

Fig. 3. The effect of FSNG and FSG on PMNs migration. The concentration of chitin and chitosan was 10mg/ml. FS was added to the suspension of chitin and chitosan, which were adjusted to the concentration of 10%. Data were shown as mean±s.d. of duplicate determination for three experiments. In the figure, a and a′ are significantly different ($p < 0.01$) from b and b′, respectively.

Invading microorganisms are coated with the polysaccharides such as glucan and mannan, which have been shown to enhance the function of phagocytes such as PMNs and macrophages (Williams *et al.*, 1986). The effect of GlcNAc and GlcN oligomers on CA for PMNs might well be expected, because even glucan (a common polysaccharide in mammals) shows biological activity for phagocytes. Some authors have suggested the existence of lectins specifically binding to GlcNAc and GlcN on the surface of macrophages (Schlesinger *et al.*, 1980; Warr, 1980). Kishimoto and Tamaki (1987) reported that application of chitin to burns prolonged the migration of lysozyme-positive cells, i.e. macrophages. Therefore, PMN (which are also lysozymepositive cells) may possess some receptors for GlcNAc and GlcN. We also found that GlcN residues were more effective chemoattractants than GlcNAc residues. However, Suzuki reported that GlcNAc6 had a greater CA for murine macrophages than GlcN6 (Suzuki *et al.*, 1986). The LD 50 of chitosan in the mouse is 10g/kg after subcutaneous administration (Mita, 1987), while that for the dog has not been clarified. Interestingly,

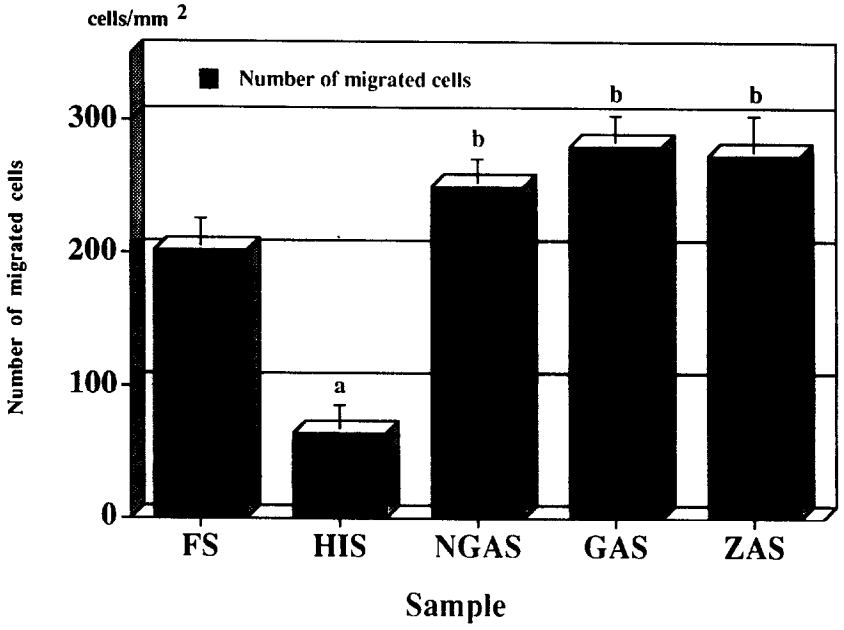

Fig. 4. The effect of NGAS and GAS on PMNs migration. Each chemoattractant was adjusted to 10%. Data were shown as mean±s.d. of duplicate determination for three experiments. The different letters in the figure mean significant difference between them ($p < 0.01$).

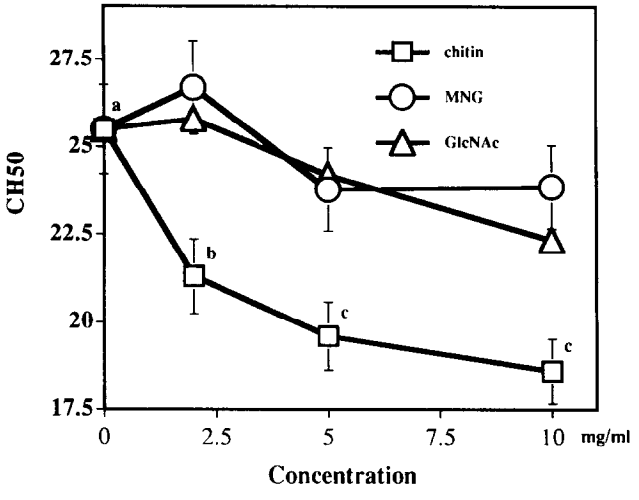

Fig. 5. The complement consuming ability of chitin, MNG and GlcNAc in canine serum. Data were shown as mean±s.d. for five experiments. The different letters in the figure mean significant difference between them: ab, ($p < 0.01$); ac, $p < 0.001$).

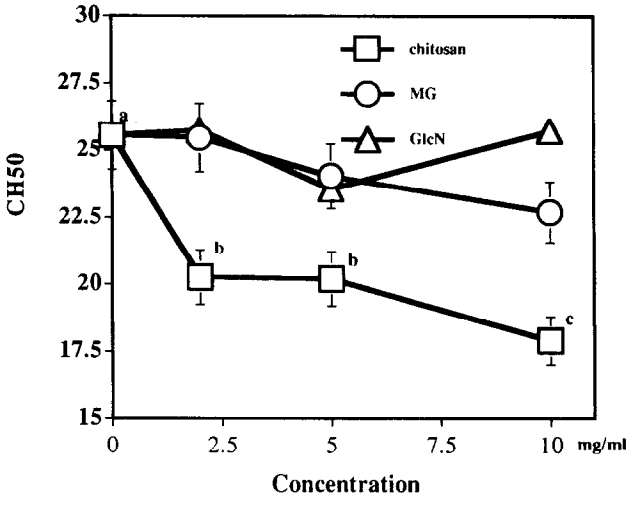

Fig. 6. The complement consuming ability of chitosan, MG and GlcN in canine serum. Data were shown as mean±s.d. for five experiments. The different letters in the figure mean significant difference between them: ab, ($p < 0.01$); ac, ($p < 0.001$).

Minami *et al.* (in press) reported that lethal pneumonia was caused by subcutaneous administration of chitosan particles (200mg/kg), with the infiltration of PMNs into the interstitial spaces and alveoli, while the same dose of chitin did not induce pneumonia and PMN migration. These reports suggest that the response to chitin and chitosan might differ between animals or between cells and might depend on the difference in the expression of receptors for GlcNAc and GlcN on the cell surface. Thus, the CA of these materials for canine macrophages needs to be determined.

We also found that chitin and chitosan particles enhanced the total complement activity of canine serum, while NGAS and GAS had a similar CA for canine PMNs to that of ZAS. These results strongly suggested that C5a in canine NGAS and GAS induced PMN migration. In our preliminary study, chitin and chitosan yielded a C5a concentration in human NGAS and GAS which was more than 40 times that in human control serum (unpublished data). In addition, it was reported that the chemiluminescence response of PMNs to chitosan was markedly enhanced by supplementation with

Table 1. Histological findings of each group

Group	No. of migrated PMNs	Perivascular infiltration of PMNs	Subcutis edema	Subcutis haemorrhage
GlcNAc	1.3*	0.0	1.0	1.0
GlcNAc6	1.3	0.6	1.0	1.0
Chitin	2.6	2.0	2.0	1.3
GlcN	2.0	1.3	1.6	2.0
GlcN6	3.0	3.0	3.0	3.0
Chitosan	3.0	3.0	2.3	2.6
Control	0.3	0.0	0.0	0.0

*The numerical values in the table are the mean degree of histological findings in each group (three dogs).

serum (Minami *et al.*, 1993). On the other hand, oligomers and monomers of GlcNAc and GlcN did not enhance total complement activity. GlcNAc and GlcN are common monosaccharides among the intracellular and intercellular oligosaccharides and glycosaminoglycans (Kornfeld & Kornfeld, 1985), so they should not enhance complement activity *in vivo*. However, MNG and MG, which are composed of GlcNAc1~6 and GlcN1~6, respectively, might be identified as foreign substances by serum, because glyco-chains (from trimer to hexamer of GlcNAc and GlcN) do not appear to exist naturally in mammalian organs (Muller-Eberhard & Schreiber, 1980). Oligosaccharides such as dextran sulfate, chondroitin sulfate and agarose, which have a higher molecular weight (20,000~60,000) than MNG and MG, are known to enhance complement activity, but low molecular weight saccharides like MNG and MG have not been reported to activate serum (Muller-Eberhard & Schreiber, 1980). Therefore, it is suggested that saccharides composed of GlcNAc and GlcN, with a molecular weight smaller than that of chitin or chitosan and larger than that of MNG or MG, might enhance complement activity.

In *in vivo* study, it was clearly demonstrated that GlcNAc6, GlcN6 and GlcN enhanced PMN migration, and GlcN6 attracted more PMNs migration than GlcNAc6. These results agreed well with the effects of GlcNAc and GlcN oligomer on PMN migration *in vitro*. Furthermore, severe subcutaneous haemorrhage and edema occurred in the GlcN6 group, however, there were few findings in the GlcNAc6 group. On the other hand, we have previously found that particles of chitin and chitosan induced directed migration of canine and bovine PMNs in the blind well chamber, and the subcutaneous implantation of chitin and chitosan induced PMN migration in dogs (Minami *et al.*, 1993; Okamoto *et al.*, 1993a). Chitosan induced more subcutis edema and haemorrhage than chitin. These results imply that GlcN6 and chitosan might not only be a direct chemotactic factor of canine PMN, but also stronger activators of the host defense system than GlcNAc6 and chitin.

This study suggested two pathways by which chitin and chitosan might enhance canine PMN migration. One is the effect of GlcNAc and GlcN residues on direct CA. We found that residues of GlcN caused more stimulation of canine PMNs than residues of GlcNAc *in vitro* and *in vivo*. Some authors have reported that chitin and chitosan were depolymerized in mammalian tissues (Yokota and Komuro, 1990; Okamoto *et al.*, 1995), and suggested that monomers and many kinds of oligomers would be derived from chitin and chitosan *in vivo*. We previously found that chitin (a polymer >90% GlcNAc and <10% GlcN) showed a stronger effect on canine PMN migration than chitosan (a polymer made up >90% GlcN and <10% GlcN) (Usami *et al.*, 1994b). Furthermore, the present study showed that the CA of GlcN oligomers, longer than GlcN6, decreased as the number of residues increased. These results suggest that there might be an optimal number of GlcN residues that enhance PMN migration, and that the number of GlcN residues of chitin would be more effective in enhancing canine PMN migration than that of chitosan. The other method by which chitin and chitosan might enhance PMN migration is via an effect on complement activity through the production of chemotactic factors such as C5a. In contrast, oligomers and monomers of GlcNAc or GlcN did not influence total complement activity. Therefore, it is suggested that complement in wound exudate activated by chitin or chitosan may induce PMN migration, but that complement activation would decrease, along with the depolymerization of chitin and chitosan.

Because chitin had a higher CA for PMNs than chitosan *in vitro* (Usami *et al.*, 1994a, b), and the complement activity contained in chitin was the same as that of chitosan, it might be expected that chitin would induce more CA than chitosan in the mammalian tissues. Okamoto *et al.* (1995) have reported that subcutaneously implanted sponge-like chitin biomaterial was absorbed after 2 weeks, while Peluso *et al.* (1994) showed that chitosan fibres persisted at 2 weeks after subcutaneous implantation. Yokota and Komuro (1990) showed that the enzymatic degradation of a chitosan film was dependent on the degree of deacetylation of the constituent chitosan and decreased with an increase of deacetylation. Therefore, because chitin would be depolymerized faster than chitosan and because GlcNAc and its oligomers have less CA than GlcN and its oligomers, both the CA and complement activity by chitin should decrease rapidly *in vivo*, with chitosan showing greater CA and complement activity than chitin.

One of the important steps in PMN migration is the upregulation of endothelial leukocyte adhesion molecules (ELA) (Bevilaequa *et al.*, 1989). There are many kinds of chemical mediators that enhance ELA, which belong to three categories: the integrin family, the immunoglobulin superfamily and the selectin family (Springer, 1990). Production of the integrin family is

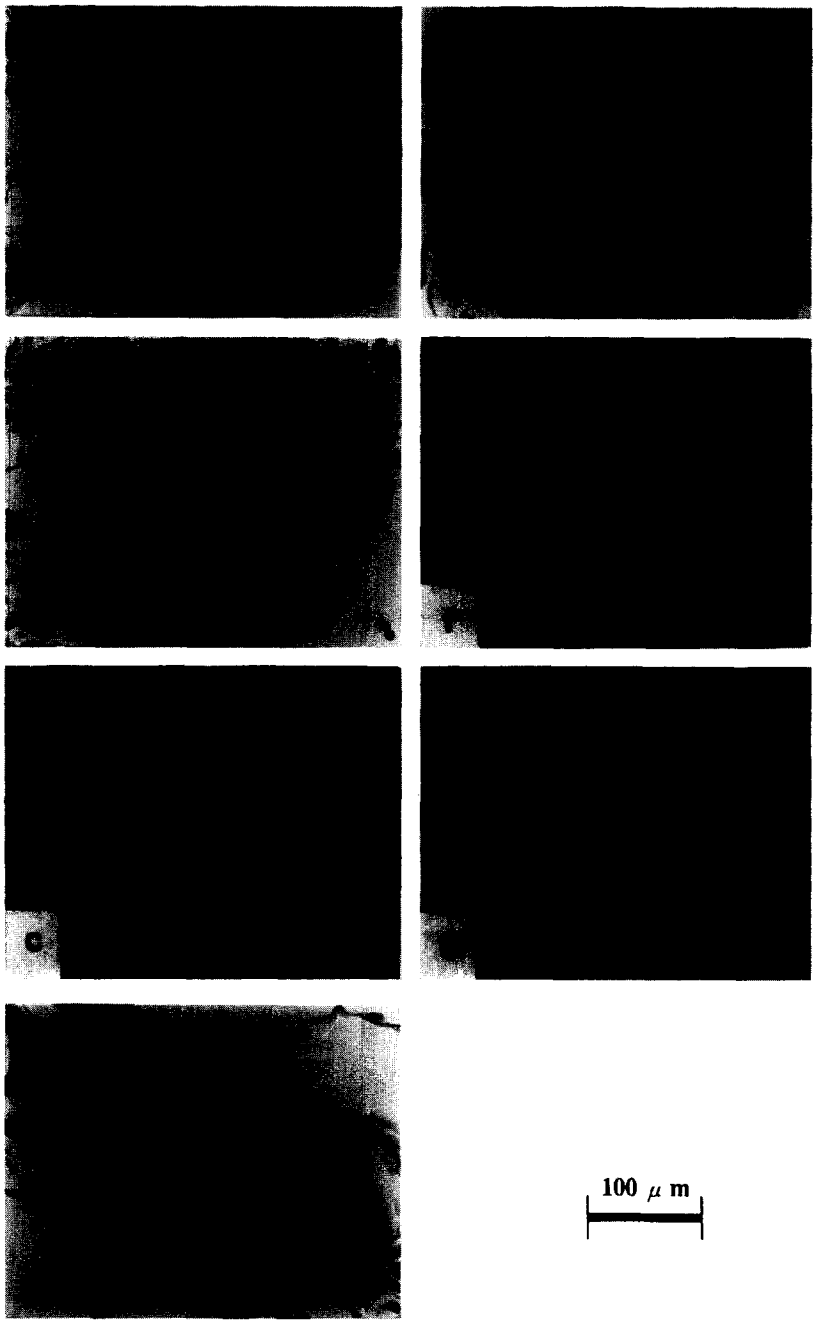

Fig. 7. Histological findings of the recovered tissues of each group: a, GlcNAc; b, GlcNAc6; c, chitin; d, control; e, GlcN; f, GlcN6; g, chitosan. Numerous PMNs migrated in the chitin, GlcN6 and chitosan groups; however, in GlcNAc and GlcNAc6 and control, little PMNs migration was observed. Arrows in each photograph mean PMN.

stimulated by C5a, IL-8, and LTB_4, while production of the immunoglobulin superfamily is stimulated by IL-1, TNF and LPS, and the production of the selectin family is stimulated by histamine, H_2O_2 and LTC_4 (Springer, 1990). We suggested that chitin and chitosan may induce the production of C5a. However, there have been no studies of the effects of chitin and chitosan on other chemoattractants. Therefore, more investigations of the substances *in vivo* and *in vitro* induced by chitin and chitosan should be performed in order to understand their effects on PMN migration.

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REFERENCES

Bevilaequa, M.P., Stengelin, S., Gimbrone, M.A. Jr & Seed, B. (1989). Endothelial leukocyte adhesion molecule 1: an

inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science*, **243**, 1160–1165.

Kishimoto, S. & Tamaki, K. (1987). Immunohistochemical and histochemical observations in the process of burn wound healing in guinea pig skin under chitin membrane dressing. *Hifuka-kiyo*, **82**, 471–479 (in Japanese).

Kornfeld, R. & Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.*, **54**, 631–658.

Minami, S., Okamoto, Y., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., Tanigawa, T., Tanaka, Y & Tokura, S. (1992). In *Advances in Chitin and Chitosan*, ed. C.J. Brine, P.A. Sandford & J.P. Zikakis. Elsevier, London, pp. 61–69.

Minami, S., Okamoto, Y., Tanioka, S., Sashiwa, H., Saimoto, H., Matsuhashi, A., & Shigemasa, Y. (1993). In *Carbohydrates and Carbohydrate Polymers*, ed. M. Yalpani. ATL Press, Mount Prospect, pp. 141–152.

Minami, S., Oh-Oka, M., Okamoto, Y., Matsuhashi, A., Shigemasa, Y. & Fukumoto, Y. Chitosan inducing haemorrhagic pneumonia in dogs. *Carbohydr. Polym.*, in press.

Mita, Y. (1987). In *Development and Application of Chitin and Chitosan*. Kogyogijutsu-kai, Tokyo, pp. 248–269 (in Japanese).

Muller-Eberhard, H.J. & Schreiber, R.D. (1980). Molecular biology and chemistry of the alternative pathway of complement. *Adv. Immunol.*, **29**, 1–53.

Okamoto, Y., Minami, S., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., Tanigawa, T., Tanaka, Y. & Tokura, S. (1992). In *Advances in Chitin and Chitosan*, eds C.J. Brine, P.A. Sandford & J.P. Zikakis. Elsevier, London, pp. 70–78.

Okamoto, Y., Minami, S., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., Tanigawa, T., Tanaka, Y. & Tokura, S. (1993). Polymeric *N*-acetyl-D-glucosamine (chitin) induce histionic activation in dogs. *J. Vet. Med. Sci.*, **55**, 739–742.

Okamoto, Y., Minami, S., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., Tanigawa, T., Tanaka, V. & Tokura, S. (1993). Application of polymeric *N*-acetyl-D-glucosamine (chitin) to veterinary practices. *J. Vet. Med. Sci.*, **55**, 743–747.

Okamoto, Y., Minami, S., Matsuhashi, A., Tanioka, S. & Shigemasa, Y. (1995). The fate of *N*-acetyl-D-glucosamine (chitin) in canine subcutaneous tissues. *J. Jap. Soc. Biomater.*, **13**, 10–14.

Peluso, G., Petillp, O., Ranieri, M., Santin, M., Ambrosio, L., Calabro, D., Avallone, B. & Balsamo, G. (1994). Chitosan-mediated stimulation of macrophage function. *Biomater.*, **15**, 1215–1220.

Schlesinger, P.H., Rodman, J.S., Doebber, T.W., Stahl, P.D., Lee, Y.C., Stowell, C.P. & Kuhlenscmidt, T.B. (1980). The role of extrahepatic tissues in the receptor-mediated plasma clearance of glycoproteins terminated by mannose or *N*-acetyl-glucosamine. *Biochem. J.*, **192**, 597–606.

Snyderman, R., Phillips, J.K. & Mergenhagen, S.E. (1971). Biological activity of complement *in vivo*. Role of C5 in the accumulation of polymorphonuclear leukocytes in inflammatory exudates. *J. Exp. Med.*, **134**, 1131–1143.

Springer, T.A. (1990). Adhesion receptors of the immune system. *Nature*, **346**, 425–434.

Suzuki, K., Tokoro, A., Okawa, Y., Suzuki, S. & Suzuki, M. (1986). Effect of *N*-acetylchito-oligosaccharides on activation of phagocytes. *Microbiol. Immunol.*, **30**, 777–787.

Usami, Y., Okamoto, Y., Minami, S., Matsuhashi, A., Kumazawa, N.H., Tanioka, S. & Shigemasa, Y. (1994). Chitin and chitosan induce migration of bovine polymorphonuclear cells. *J. Vet. Med. Sci.*, **56**, 761–762.

Usami, Y., Okamoto, Y., Minami, S., Matsuhashi, A., Kumazawa, N.H., Tanioka, S. & Shigemasa, Y. (1994). Migration of canine neutrophils to chitin and chitosan. *J. Vet. Med. Sci.*, **56**, 1215–1216.

Warr, G.A. (1980). A macrophage receptor for (mannose/glucosamine)-glycoproteins of potential importance in phagocytic activity. *Biochem. Biophys. Res. Commun.*, **93**, 737–745.

Williams, J.D., Topley, N., Alobaidi, H.M. & Harber, M.J. (1986). Activation of human polymorphonuclear leukocytes by particulate zymosan is related to both its major carbohydrate components: glucan and mannan. *Immunol.*, **58**, 117–124.

Yokota, C. & Komuro, T. (1990). Studies on the degradation of chitosan films by lysozyme and release of loaded chemicals. *Yakugaku-zasshi*, **110**, 442–448.