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Effect of *N*-acetyl-D-glucosamine and D-glucosamine oligomers on canine polymorphonuclear cells in vitro

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Abstract

The effect of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) oligomers on the activation and viability of canine polymorphonuclear cells (PMNs) was evaluated. Checkerboard assays of both oligomers revealed that the GlcNAc oligomer induced chemotactic migration, while GlcN oligomer induced chemotactic and chemokinetic migration. The GlcN oligomer showed about twice the chemotactic activity of the GlcNAc oligomer, and induced slight enhancement of the chemiluminescence response (CL) of canine PMNs to zymosan, as well as shortening of the peak time of the CL response compared with the control and the GlcNAc oligomer. Neither oligomer showed cytotoxicity for canine PMNs at a concentration of 100 μg/ml. These results suggest that GlcNAc and GlcN oligomers might be activating factors for canine PMN in vivo.

Keywords: Polymorphonuclear; *N*-acetyl-D-glucosamine; D-glucosamine oligomers

1. Introduction

We have demonstrated that chitin and chitosan, which are polymers of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN), have biological effects that include acceleration of wound healing and chemotactic activity (Minami et al., 1993; Okamoto et al., 1993; Usami et al., 1994b). Numerous polymorphonuclear cells (PMNs) accumulate, around subcutaneously implanted chitin and chitosan in dogs, and most of them were fresh (Minami et al., 1992; Okamoto et al., 1993). Chitin and chitosan are known to be depolymerized by lygozymes which, are widely found in mammalian tissues and cells, and thus would be degraded into GlcNAc and GlcN oligomers and monomers in vivo (Berger and Weiser, 1957; Minami et al., 1992; Okamoto et al., 1993).

GlcNAc and GlcN various biological activities including a bactericidal effect, macrophage activation, and an antitumor effect in mice (Suzuki et al., 1986; Tokoro et al., 1988; Tokoro et al., 1989). These findings strongly suggest that GlcNAc and GlcN oligomers influence PMNS. Recently, we also showed that GlcNAc hexamer was a strong attractant for canine PMNs (Usami et al., 1997). However, it has not been detertnined whether the effects of both oligomers on PMN migration are as a result of chemokinesis or chemotaxis.

The present study investigated the effects of GlcNAc and GlcN oligomers on the activation and viability of canine PMNS.

2. Experimental

2.1. Materials

Oligomers: Oligomers of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) were obtained by depolymerization and partial acid hydrolysis of chitin and chitosan (Ruply, 1964; Horowitz et al., 1957) prepared from snow crab shell. The oligomers were a mixture of GlcNAc1~GlcNAc6 and GlcN1~GlcN6, respectively (Yaizu Suisankagaku Industries, Japan). Each percentage of compositions was analyzed with HPLC and shown as follows: a mixture of GlcNAc oligomer — GlcNAc6, 4.6; GlcNAC2, 30.0; GlcNAc3, 26.8; GlcNAC4, 19.9; GlcNAc5, 12.9; and GlcNAC6, 5.5. a mixture of GlcN oligomer — GlcN, 26.9; GlcN2, 21.4; GlcN3, 16.8; GlcN4,

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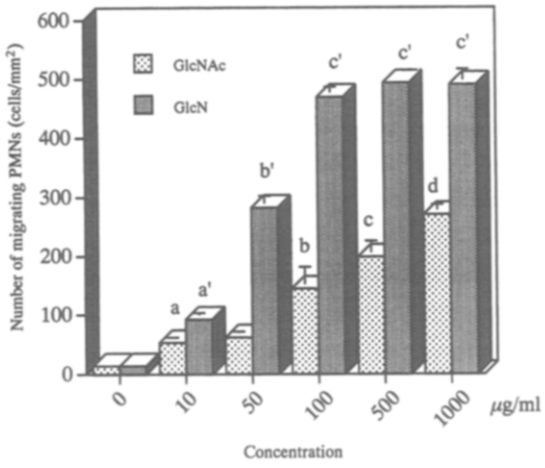

Fig. 1. The effect of the GlcNAc and GlcN oligomers on canine PMN migration with a modified Boyden chamber method. 0:HBSS, GlcNAc: GlcNAc oligomer, GlcN: GlcN oligomer. Data are displayed as the mean ± S.D. for five experiments. The different letters in the figure indicate a significant different between them. ab, ac, ad ($p < 0.05$), a'b', a'c' ($p < 0.01$).

4.1; GlcN5, 3.2; GlcN6, 1.8 (Sakai, 1987). The oligomers were diluted with Hank's balanced salt solution (HBSS) and the solutions were sterilized by passage through a cellulose nitrate membrane filter (0.45 μm) before use in this study.

2.2. Blood collection

Peripheral blood was collected from five mongrel dogs aged 1–3 years (three males and two females).

2.3. Separation of PMNs

Separation of canine PMNs from the collected blood was done by density gradient centrifugation (S.G. = 1.082), as reported previously (Usami et al., 1994b).

2.4. Measurement of canine PMN migration

The migration of canine PMNs in response to GlcNAc and GlcN oligomers was measured using the blind well chamber method (Nuclepore, U.S.A.), as reported previously (Usami et al., 1994a)

In brief, 200 μl of GlcNAc or GlcN solution was placed into the lower chamber and a 5 μm pore-sized polycarbonate filter (Neuro Probe, U.S.A.) was positioned between the lower chamber and the upper chamber. Then 200 μl of cell suspension was added to the upper chamber and incubated at 37°C for 60 min. Each filter was then removed, air-dried, fixed in methanol, stained with light Giemsa solution, and mounted on a glass slide. The migrating cells which had completely passed through the pores and attached to the lower surface of the filter were counted in 30 randomly selected oil immersion fields under a microscope at a magnification of 1000. The results were expressed as the number of PMNs per mm^2 of filter surface.

2.5. Chemiluminescence response of canine pmns

The chemiluminescence response (CL) was measured with a Lumat LB-9501 (Berthold, Germany) as follows. The 100 μl of blood sample was preincubated with 400 μl of GlcNAc or GlcN oligomer solution (125 μg/ml in HBSS) at 37°C for 15 min. Then 20 μl of luminol (2 mg/ml) was added to the cuvette and incubated another 5 min. One minute after the start of CL measurement, 50 μl of Zymosan (10 mg/ml) was added to the cuvette as a stimulant. The CL response was measured for 15 min and the CL intensity per 1000 PMNs (CL index) was calculated by the following equation:

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

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

2.6. MTT assay

The viability of canine PMNs was determined using the previously described MTT assay (Mosmann, 1983). Briefly, 100 μl of PMN suspension (2×10^6 cells/ml) was inoculated into 96-well microtiter plates. Then 10 μl of the GlcNAc or GlcN oligomer solution was added to each well and incubated at 37°C for 2–22 h. MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, St Louis, MO] was prepared at 5 mg/ml in 0.1 M PBS without calcium and magnesium (Nissui, Quality Biological, Japan) and was stored at 4°C. After an appropriate time, 25 μl of MTT solution was added to each well and incubated at 37°C for 2 h. Extraction buffer was prepared with 20 g of sodium lauryl sulfate (SDS, Bio-Rad, USA) in 47.5 ml of H_2O and 47.5 ml of *N,N*-dimethylformamide (DMF, Aldrich, USA) and titrated with HCl to pH 4.7. After incubation, 100 μl of extraction buffer was added to each well to solubilize the NM-forinazan product, and then pipetted to mix the product. The absorbance of each well was measured at 550 nm with a microplate reader (Model EAR 340 AT, SLT-labinstrument, Austria).

3. Statistical analysis

Statistical analysis was performed using Student's *t*-test. Migration and MTT assays were, respectively, carried out in duplicate or triplicate and repeated five times. CL assays were repeated four times.

4. Results

The number of canine PMNs migrated in response to GlcNAc and GlcN oligomers is shown in Fig. 1. The

Table 1
A checkerboard assays on the canine PMNs migration to the GlcNAc and GlcN oligomers

Concentration in the upper well (μg/ml)		Concentration in the lower well (μg/ml)		
		0	500	500
GlcNAc oligomer	0	18 ± 3[a]	62 ± 10[b]	198 ± 27[c]
	50	8 ± 2	15 ± 2	60 ± 10[b]
	500	8 ± 2	9 ± 2	21 ± 3
GlcNAc oligomer	0	17 ± 3[a']	282 ± 20[b']	493 ± 8[c']
	50	10 ± 3	90 ± 9[c']	260 ± 30[b']
	500	20 ± 3	75 ± 2	109 ± 16

The checkerboard assays of the effect of GlcNAc and GlcN oligomers on canine PMN migration with modified Boyden chamber method.
Data are displayed as mean ± S.D. for five experiments.
The different letters in the figure indicate a significant difference between them. ab, bc, a'b', a'c', a'd' ($p < 0.01$).

PMN migration to the GlcNAc oligomer was greater than that observed to HBSS and increased in a concentration-dependent manner. The GlcN oligomer induced more PMN migration than the GlcNAc oligomer at each concentration. A rapid concentration-dependent increase of PMN migration to the GlcN oligomer was observed at concentrations of 10, 50 and 100 μg/ml, but the number of migrated PMN displayed a plateau at 100, 500, and 1000 μg/ml. The GlcN oligomer induced about 1.5- to 4.0-fold more migration than the GlcNAc oligomer.

Table 1 shows checkerboard assays of PMN migration to GlcNAc and GlcN oligomers. Canine PMNs incubated in the presence of a positive GlcNAc or GlcN oligomer gradient exhibited markedly increased migration. The maximum migratory activity was obtained when the concentration of the GlcNAc or GlcN oligomer in the lower and upper wells was 500 and 0 μg/ml, respectively, and was about 11- and 29-fold greater than when the oligomers were absent from both wells. However, canine PMNs incubated in the presence of a negative oligomer gradient showed less migration. Thus, the canine PMNs were confirmed to be attracted to the GlcNAc and GlcN oligomers chemotactically. With a same concentration of the GlcN oligomer in the upper and lower wells, when the concentration was increased in both wells, the number of migrated PMNs increased significantly ($p < 0.01$). But the GlcNAc group did not show significant change. This shows that GlcN oligomer induced chemokinesis of canine PMNs, but not the GlcNAc oligomer.

The effects of the oligomers on CL by canine PMNs are shown in Figs. 2–4. The CL index did not change when the GlcNAc oligomer was added to the PMNs, but it reached 1.3-fold of the control level after preincubation with the GlcN oligomer. This oligomer also slightly reduced the peak time of the CL index compared with the control.

The MTT assay of canine PMNs incubated with the oligomers showed a time-dependent decline of the absorbance in all three groups (Fig. 5). There was a plateau for 6 h of incubation, and then a gradual decrease for 22 h.

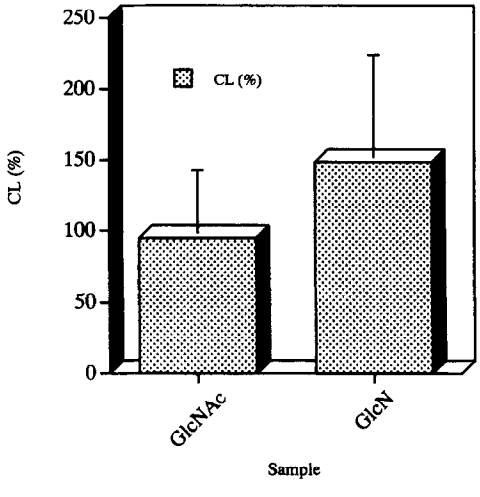

Fig. 2. The effect of the GlcNAc and GlcN oligomers on the peak count (RLU/s/1000) of canine PMN CL response. GlcNAc:GlcNAc oligomer, GlcN: GlcN oligomer. Data are displayed as a percentage of the control (mean ± S.D.). CL (%); The peak count in preincubation with GlcNAc (GlcN) oligomer/the peak count in preincubation with HBSS.

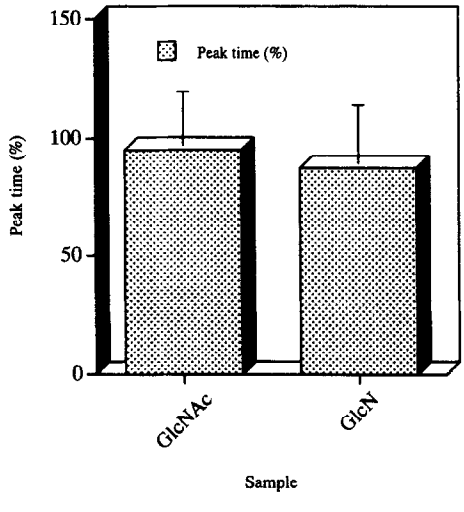

Fig. 3. The effects of GlcNAc and GlcN oligomers on the peak time of canine PMN CL response. GlcNAc:GlcNAc oligomer, GlcN:GlcN oligomer. Data were shown as percentage of the control (mean ± S.D.). Peak time (%); The peak time in preincubation with GlcNAc (GlcN) oligomer/the peak time in preincubation with HBSS.

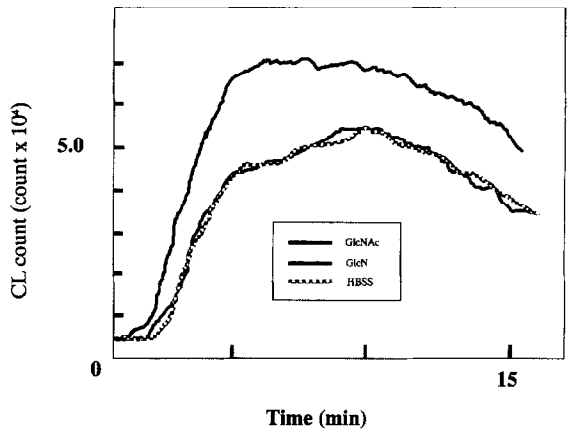

Fig. 4. The effect of GlcNAc and GlcN oligomers on canine PMN CL response. HBSS: Control, GlcNAc:GlcNAc oligomer, GlcN:GlcN oligomer.

5. Discussion

The GlcNAc and GlcN oligomers were both shown to attract canine PMN with the GlcN oligomer exhibiting a greater attraction. PMNs migration to GlcN oligomer reached a plateau when the oligomer concentration was between 100-1000 μg/ml, but GlcNAc oligomer induced dose-dependent PMNs migration. In a preliminary experiment, GlcN oligomer did not show any cytotoxic effect on canine PMNs (2×10^5 cells per mg of GlcN oligomer, data not shown). This phenomenon has also been reported for LTB4 and C5a (Thomsen and Jensen, 1991), and PMN migratory activity shows a plateau at a high concentrations of such chemotactic substances. Some authors have reported that PMN migration may depend on both the number and the sensitivity of receptors, as well as the concentration of chemotactic factors (Snyderman and Goetzl, 1981). Macrophages are lysozymes-containing cells and have receptors

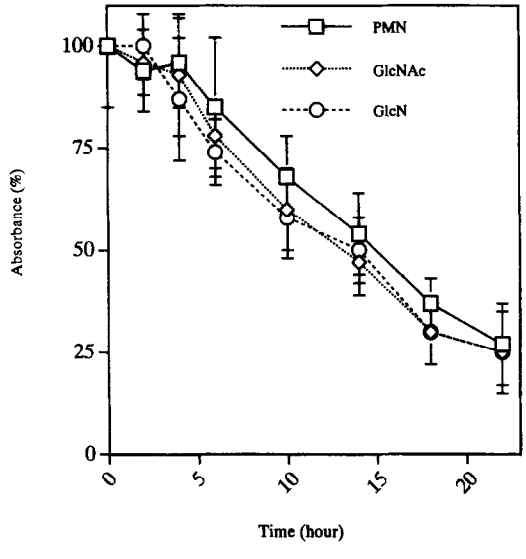

Fig. 5. The effect of GlcNAc and GlcN oligomer on cytotoxicity for canine PMN. PMN: Control, GlcNAc:GlcNAc oligomer, GlcN:GlcN oligomer. Data were displayed are mean ± S.D. for five experiments.

binding GlcNAc and GlcN (Warr, 1980). Our results suggest that canine PMNs might recognize GlcNAc and GlcN oligomers, but are more sensitive to the latter. However, there are no data regarding receptors for GlcNAc and GlcN on canine PMN, so further investigation is necessary.

The checkerboard assay proved that GlcNAc and GlcN oligomers were chemotactic factors for canine PMNs and that GlcN oligomer was also a chemokinetic factor. Furthermore, GlcN oligomer was found to be a more effective chemotactic factor than GlcNAc oligomer. Chitin (90% GlcNAc) induced both chemotactic and chemokinetic migration of canine PMNS, but chitosan (80% GlcN) enhanced only chemotactic migration (Usami et al., 1994b). The chemotactic response of macrophage to chitosan as higher than that to GlcN (Peluso et al., 1994). Migrating cells had a optimum concentration of each polymer or oligomer of GlcNAc and GlcN to show the higest migratory activity (Suzuki et al., 1986; Peluso et al., 1994). These findings suggest that the effect of GlcNAc and GlcN on canine PMNs is related to the chain length of these oligomers and would change when the GlcNAc and GlcN chains are depolymerized.

One of the important roles of PMNs in host defenses is phagocytosis (Makimura and Sawaki, 1992) phagocytize micro-organisms and foreign materials. GlcN oligomer slightly enhanced the CL response of canine PMN and reduced the peak time of the CL index. Many kinds of agents, such as lipopolysaccharide (LPS) (Henricks et al., 1983) and tumor necrosis factor (Bajaj et al., 1992), have a priming effect and enhance PMN migration both in vitro and in vivo. As the PMN migration assay revealed that GlcN oligomer showed more effective chemotactic activity than GlcNAc oligomer, our results suggest that GlcN oligomer might also promote PMN function in vivo more strongly than GlcNAc oligomer.

Both oligomers showed no cytotoxicity for the canine PMNs. Chemotactic factors such as LTB4 and C5a also did not induce cytotoxicity at concentrations with chemotactic effect (Thomsen and Jensen, 1991), and are metabolized into non-chemotactic compounds by cell organelles and the cell membrane. GlcNAc and GlcN oligomers may be phagocytized or degraded into monomers by PMN lysozymes. Furthermore, many kinds of saccharides are utilized by glucolytic system in inflammatory cells and other cells, and GlcNAc and GlcN are metabolized by this system (Brzezinski and Serhan, 1990; Robinson and Stirling, 1968). Thus, these oligomers might be utilized by canine PMNs as a source of energy. Accordingly, GlcNAc and GlcN oligomers might not continue to stimulate PMNs and thus might not induce cytotoxicity.

The present study showed that GlcNAc and GlcN oligomers enhanced PMN migration without cytotoxicity and GlcN oligomer was effective in promoting CL. Chitin and chitosan, polymers of GlcNAc and GlcN, also enhance canine PMN migration, chemotactically (Usami et al., 1994b). These results suggest that chitin and chitosan may

induce greater migration of canine PMNs in vivo after being depolymerized by lysozymes into GlcNAc and GlcN oligomers in the subcutaneous tissue (Berger and Weiser, 1957; Minami et al., 1992; Okamoto et al., 1993). PMN migration is one of the important steps in repair of tissue damage (Snyderman and Goetzl, 1981). These results, therefore, support the effect of chitin and chitosan as wound healing accelerants, for which they have been popular biomaterials in the medical and veterinary medical fields. However, the effect of GlcNAc and GlcN oligomers on wound healing is not clear. More investigations about the in vivo effects of these oligomers on many various tissues and cells should be performed in the future.

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