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Systemic effect of chitin after intravenous administration to dogs

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The systemic effect of chitin and chitin oligomer after intravenous administration was investigated in dogs by determining the chemiluminescence (CL) response and the white blood cell count (WBC). Chitin oligomer (2 mg/kg) and physiological saline (5 ml) did not have a systemic effect. However, in the dogs injected with chitin, WBC decreased significantly from 1 to 4 h after injection and then increased gradually to 1.4 times the pre-injection level at 72 h, while CL was significantly increased 1–2 h after injection. Copyright © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Chitin is a straight polymer of *N*-acetyl-D-glucosamine (GlcNAc) or β 1-4 linked polysaccharide of 2-acetamide-2-deoxy-D-glucose. We have developed chitin remedies for veterinary use and have demonstrated its superior biocompatibility and acceleration of wound healing (Okamoto *et al.*, 1993). When chitin was applied to wounds, it showed complete biodegradation within 2 weeks (Okamoto *et al.*, 1995). In animal tissue, chitin is degraded by lysozyme into chitin oligomer and is further degraded into GlcNAc by β-*N*-acetylglucosaminidase and other enzymes (Kifune, 1994). The monomer, GlcNAc, exists in extremely high levels in the body and is utilized for heat production, so it is seldom recognized as a foreign substance (Kifune, 1994).

Two possible routes for absorption of subcutaneously implanted chitin can be suggested. One is secondary absorption after biodegradation into chitin monomers and oligomers, while the other route is direct absorption of chitin into the blood vessels through the lymph system. It is very important clinically to determine whether the absorption of polymers and/or oligomers via the lymph system will cause problems like thrombosis or any other undesirable effects.

In the present study, the effects of direct polymer and oligomer migrations into vessels on the blood components and chemiluminescence (CL) of polymorphonuclear cells (PMN) were investigated.

EXPERIMENTAL

Animals

Twelve adult male mongrel dogs aged 1–3 years and weighing 8–14 kg, which were normal on physical and laboratory examination, were used in this study. The dogs were divided into four groups of three each, i.e. a chitin group, a chitin oligomer group, a latex group, and a physiological saline (control) group.

Drugs

Chitin suspension

Commercial squid pen β-chitin flakes (Nippon Suisan Co. Ltd, Tokyo) were purified from Neon flying squids (*Ommastrephes bartrami*). This chitin was 9% deacetylated and had an average molecular weight of over 100 000. The chitin flake was pulverized into 3 μm particles using a mill (Ube Industries Ltd., Japan, CF-400); the resulting fine powder was sterilized with ethylene oxide gas, and suspended in physiological

saline at a concentration of 10 mg/ml. Dogs were administered 5 ml of various dilutions of this suspension in physiological saline.

Chitin oligomer
A chitin oligomer mixture was purchased from Yaizu Suisankagaku (Yaizu, Japan). This mixture was obtained by depolymerization and partial acid hydrolysis of chitin prepared from snow crab shell. The main component of this chitin oligomer was GlcNAc6. and small amounts of GlcNAc1-GlcNAc5 were also present. The mixture was dissolved in physiological saline at a concentration of 10 mg/ml. Then an aliquot of this solution diluted to 5 ml with physiological saline was given to each dog. The final solution for each dog was sterilized by Millipore filtration before use.

Latex suspension
Latex particles (polybead latex microparticles, 04-0171-34, particle size 3 μm) were purchased from Funakoshi Co. Ltd.(Tokyo) and were suspended in physiological saline at a concentration of 10 mg/ml. Then an aliquot of this suspension diluted to 5 ml with physiological saline was given to each dog, after being sterilized by Millipore filtration.

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

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

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

Flow cytometry
Flow cytometry of lymphocytes was performed with an EPICS-XL (Coulter, USA). The excitation wavelength was 488 nm, and fluorescein isothiocyanate (FITC) was used as the fluorochrome. A rat anticanine CD8 antibody (Serotec Inc., Oxford) was used as the primary antibody and a goat anti-rat IgG labeled with FITC (E Y Laboratories, Inc., California) was used as the secondary antibody.

METHODS

Administration of drugs

Five ml of suspension (chitin or latex) or solution (oligomer) was administered at a dose of 2 mg/kg into the cephalic vein. In the control group, 5 ml of physiological saline was administered into the cephalic vein.

Blood collection and examination

Five ml of blood was collected from the jugular vein into a tube with heparin (10 IU/ml). A standard complete blood count was performed on 1 ml. Two ml of blood was used for the CL assay and the remaining blood was centrifuged to separate plasma and used for biochemical analyses. The red blood cell count (RBC), white blood cell count (WBC), differential WBC, serum total protein (TP), packed cell volume (PCV), and hemoglobin concentration (Hb) were measured in a routine manner. The mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV) were calculated from the values of RBC, Hb, and PCV, respectively. The reticulocyte count was determined after staining with cresyl brilliant blue and expressed as cells per 1000 RBC. In all groups except for the chitin oligomer group, blood was collected before the administration of the agent (Pre), as well as 1 h, 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, 72 h, 120 h, and 168 h after administration. In the chitin oligomer group, blood was collected Pre, as well as 1 h, 2 h, 4 h, 6 h, 24 h, and 72 h after administration.

The following biochemical tests were performed using a Cobas Ready dry chemistry system (Japan Roche, Tokyo): D-glucose (Glu), total cholesterol (CHOL), blood urea nitrogen (BUN), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), 7-glutamyltransferase (GGT), calcium (CALC), creatinine phosphokinase (CK), creatinine (CREA), and lactate dehydrogenase (LDH). Cortisol was examined by enzyme immunoassay (Cortisol[BMY], Boehringer Mannheim, Tokyo) using a spectrophotometer (Hitachi, model 100-22, Tokyo) at a wavelength of 405 nm.

Chemiluminescence (CL) assay

Chemiluminescence (CL) was determined using a Lumat LB-9501 (Berthold, Germany) as described by Makimura and Sawaki (1992). In brief, blood dilute fivefold with HBSS was incubated at 37 °C for 15 min in the cuvette, and then 20 μl of luminol (2 mg/ml) was added. After incubation for 5 min more, the CL

intensity was measured. The baseline value was determined for 1 min, and 50 μl of zymosan (10 mg/ml) was then added to the cuvette as a stimulator. The CL response was measured for 15 min and the peak count was determined (CL value). The CL intensity per 1000 PMNs (CL index) was calculated by the following equation:

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

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

Flow cytometry

At 72 h after chitin injection, blood was collected and then 1 μl of primary antibody (1:200) was added to 100 μl of blood. The mixture was incubated for 30 min at room temperature and then washed twice with PBS. Then 1 ml of the secondary antibody (1:400) was added to the washed blood and incubated in ice water in a dark room. After rinsing once with PBS, the RBCs were hemolyzed in ammonium chloride solution. After rinsing twice, the cells were suspended in 1 ml of PBS. The cell suspension was injected into the flow cytometer and forward light scatter (FS) and side scatter (SS) were automatically measured for 500 cells. From the histogram thus generated, the lymphocyte gate was set and FITC-positive cells were counted in the gate.

In order to assess CD8-positive cells, the same analysis was performed for 5000 cells, and it was determined whether the positive cells remained in the same position in the gate.

STATISTICAL ANALYSIS

Statistical analysis was performed by the paired t test with Stat View 4.5 software (Abacus Concepts, USA) or by the unpaired t test with DA Stat 1.0 software (Nifty Serve, USA) for Macintosh.

RESULTS

Blood tests

Chitin and the latex groups

The RBC, Hb and PCV decreased during 1–6 h after injection in the chitin and latex groups compared to the control group. However, the changes were not significant. The MCV, MCHC, and reticulocyte count did not change in all groups. On the other hand, the WBC decreased significantly during 1–4 h after injection in the chitin group compare to the others (Fig. 1). In the chitin group, WBC gradually increased after 4 h and was 1.4 times the pre-injection value at 72 h (Fig. 2). These changes of the WBC were largely due to changes of segmented neutrophils, although the lymphocyte count also increased at 72 h (Fig. 2). In the latex group, WBC did not change during the experimental period, but the number of neutrophils increased from 4 to 24 h (Fig. 1). None of the biochemical markers (Table 1) or the cortisol level (Table 2) showed any changes during the experimental period.

Chitin oligomer and the control groups

In the chitin oligomer group, WBC showed a transient, but significant, increase at 24 h (Table 3), but there were no significant changes of the other blood parameters during the whole experimental period. In

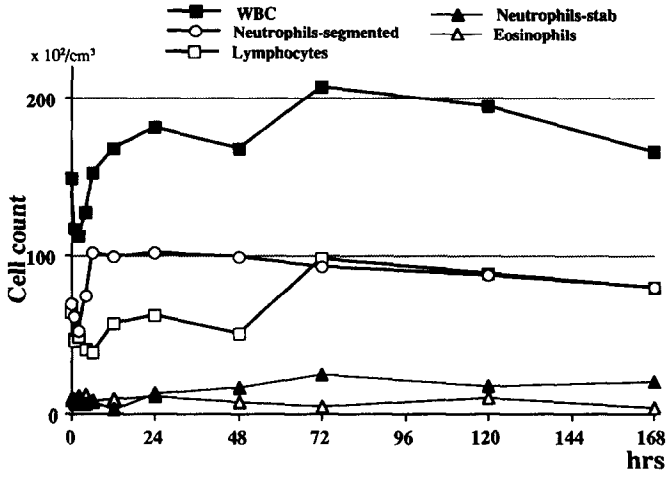

Fig. 1. The white blood cell count (WBC) in each croup. *Values were significantly lower than before injection (0 h) in the chitin group at 1, 2, and 4 h after injection ($p < 0.05$). **WBC are expressed as the mean % and standard error in each group. Individual data were converted to % relative to the initial WBC (0 h).

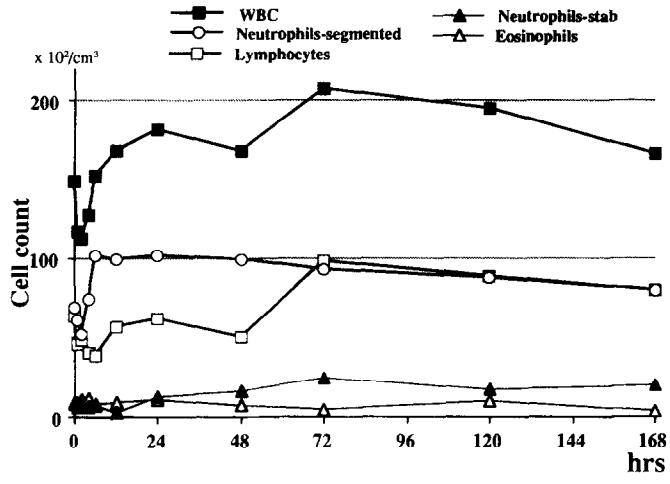

Fig. 2. White blood cell count (WBC) and differential leukocyte counts in the chitin group.

Table 1. The results of biochemical analyses in the chitin group

Item	Blood collection time (h) before (0) and after chitin injection (mean±s.d.)					
	0	2	12	24	72	168
GLU (mg/dl)	86.0±3.46	85.3±7.57	77.7±4.93	79.0±7.94	73.3±10.69	73.7±15.1
CHOL(mg/dl)	97.7±22.4	97.0±26.9	112.0±34.1	108.7±31.6	101.0±51.2	95.3±47.5
BUN (mg/dl)	21.6±8.74	22.3±8.62	11.3±1.15	16.0±7.81	17.0±6.56	24.3±4.51
AST (IU/l)	16.3±1.53	15.0±3.61	14.3±2.52	13.3±4.93	14.0±6.93	15.3±6.66
ALT (IU/l)	36.3±19.5	33.0±20.7	38.3±16.0	35.7±16.0	35.7±24.0	32.0±19.0
ALP (IU/l)	66.7±22.3	61.7±18.5	63.7±8.1	65.0±8.5	57.7±5.0	54.7±7.23
LDH (IU/l)	235.3±134.1	188.3±27.1	234.7±91.5	138.3±12.5	256.7±142.7	174.0±31.2
CREA (mg/dl)	0.87±0.153	0.97±0.379	0.83±0.153	0.80±0.153	0.73±0.058	0.73±0.115
CALC (mg/dl)	10.3±0.17	10.2±0.31	9.9±0.40	10.6±0.15	11.3±2.12	10.5±1.34
GGT (IU/l)	26.0±1.4	26.5±3.5	25.7±0.6	25.0±0	24.7±0.6	25.0±0
CK (IU/l)	192.0±95.8	162.7±88.6	164.3±31.0	149.0±16.5	196.0±31.0	132.3±55.3

Table 2. Cortisol levels in the chitin and latex groups

Group	Blood concentration of cortisol (μg/dl, mean±s.d.)				
	0	1	2	6	24[a]
Chitin	1.0+0.9	2.1+1.3	1.6±0.4	1.5±0.6	2.3±1.0
Latex	2.5±1.0	2.8±0.1	2.7±0.6	2.9±0.2	2.1±0.5

*Blood collection time (h) before (0) and after chitin or latex injection.

Table 3. Chemiluminescence (CL) index and white blood cell count (WBC) in the chitin oligomer and control groups

Item	Blood collection time (h) before (0) and after injection (mean±s.d.)						
	0	1	2	4	6	24	72
				Latex group			
CL index	2933±46	3090±260	2580±211	3203±550	2777±210	2127±209	2243±255
WBC	104±21	92±16	101±8	99±11	101±8	149±6[a]	109±21
				Control group			
CL index	2856±31	3010±85	3120±223	3090±320	2910±40	3127±150	2840±56
WBC	110±28	103±35	123±30	130±45	125±23	118±16	115±18

[a]Significant difference ($p < 0.05$) between 0 h (before injection) and 24 h.

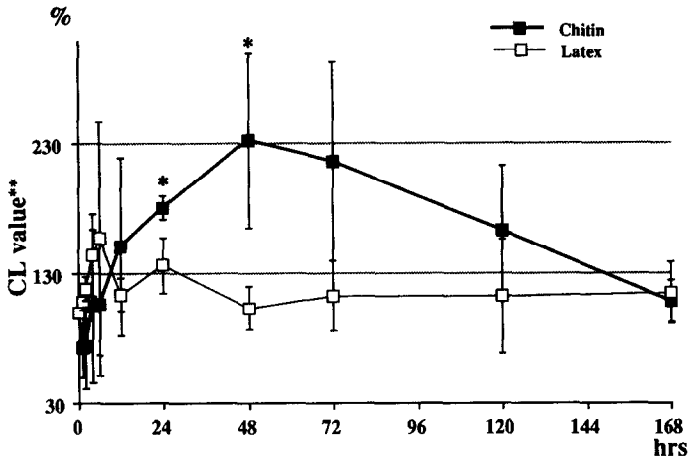

Fig. 4. Long-term chances of chemiluminescence (CL) values in the chitin and latex groups. *Values are significantly higher than before injection (0 h) in the chitin group at 24 and 48 h after injection ($p < 0.05$). **CL values are expressed as the mean % and standard error in each group. Individual data were converted to % relative to the initial CL peak count (0 h).

the control group, there were no significant changes of WBC and other blood parameters during the whole experimental period (Table 3).

Chemiluminescence

Chitin and the latex groups

The CL value decreased significantly during 1–2 h after injection in the chitin group (Fig. 3), and increased significantly at 24 and 48 h (Fig. 4). On the other hand, in the latex group the CL value rose to 1.5 times the pre-injection level at 6 h after injection (Fig. 3), and then returned to the pre-injection level (Fig. 4). There were no significant variations of the CL index in the chitin and latex groups (Fig. 5).

Chitin oligomer and the control groups

There were no significant changes throughout the experimental period (Table 3).

Flow cytometry

Figure 6 shows the number of CD8-positive cells in the chitin group and the control group. The percentage of CD8-positive cells in the chitin group and the control group was 26.6±5.09% and 25.0%, respectively, showing no significant difference.

DISCUSSION

In the present study, intravenously administered chitin induced a significant decrease of the WBC up to 4 h after injection and the CL value also decreased as the acute response. These reactions were probably caused by chitin particles, because the other groups showed no such responses. We have already examined systemic activation by chitin after it was administered subcutaneously. There were no significant changes even

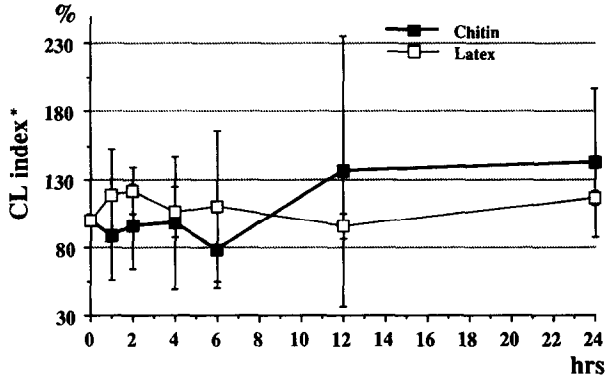

Fig. 5. Chemiluminescence (CL) indexes in the chitin and latex groups. *The CL index is expressed as the mean % and standard error in each group. Individual data were converted to % relative to the initial CL peak count (0 h).

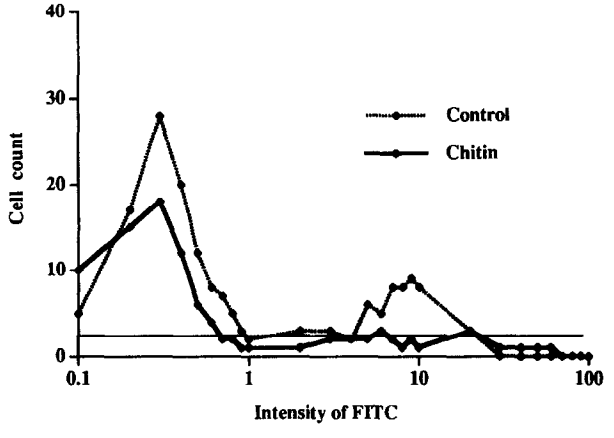

Fig. 6. The cell number of CD8-positive lymphocyte at 72 h after chitin injection. Chitin: blood at 72 h after administration of chitin suspension (2 mg/kg). Control: blood of normal dog.

in the dogs injected with 10 and 100 mg/kg subcutaneously (data not shown). From the present finding that 2 mg/kg of chitin intravenously caused some systemic reaction, it seems unlikely that direct migration of 2 mg/kg of chitin particles into the blood occurred after 100 mg/kg was given subcutaneously.

The RBC parameters decreased spontaneously in the chitin and the latex groups, but not in the chitin oligomer and control groups. These responses were probably caused by water insoluble particles trapped in liver, spleen and other reticuloendothelial organs. It has been reported that a colloidal substance will enhance or reduce reticuloendothelial function (Takahashi, 1992). Hypersplenism causes a decrease of RBC and WBC with normal erythrocytic parameters (Nakamuta *et al.*, 1993, Sawashima *et al.*, 1990). The responses to chitin and latex in the present study closely resemble these reports, suggesting that chitin and latex particles are trapped in the spleen and degraded by phagocytosis. However, histological investigation of the liver and spleen will be required to confirm this. On the other hand, all biochemical parameters including the cortisol level, did not show any changes during the experiment, suggesting that the responses induced by chitin particles did not cause any serious problems for the animals.

We have previously reported that the mechanism of wound healing acceleration by chitin involves IL-1 production (Tanigawa *et al.*, 1992), PGE_2 production (Minami *et al.*, 1995), and promotion of PMN chemotaxis (Usami *et al.*, 1994). In our recent study, chitin was found to activate complement in the serum (data not shown). IL-1 (Bachert *et al.*, 1995, Weill *et al.*, 1995) and activated complement, especially C5a (Monk *et al.*, 1994), cause upregulation of adhesion receptors on the vascular endothelium and also on the surface of PMNs. These could cause many PMNs to adhere to the endothelium and thus decrease the WBC, especially the neutrophil count and the CL value as occurred in the present study.

Chitin also induced leukocytosis, an increase of the CL index and lymphocytosis at 24 h and 48 h as delayed responses. It is known that deacetylated chitin induces an increase of colony stimulating factor in mice (Iida *et al.*, 1987), but there are no data about chitin. IL-1 increases the number of T cells (Lindemann *et al.*, 1995), but not B cells (Collins and Oldman, 1995). IL-1 also supports T cell differentiation to killer cells (Dett *et al.*, 1991). The flow cytometry data revealed no apparent increase in the number of CD8-positive cells. This would mean that intravenous chitin does not increase the number of T cells. Leukocytosis and the increasing CL index are signs of inflammation and are caused by the activation of serum complement by chitin. In the clinical setting, such systemic activation, especially of neutrophils, would be useful for patients in an immunosuppressed state due to various surgical procedures. However, further investigations are required on the activation of complement in animals of the chitin and latex groups.

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