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Immunostimulating Effect of Chicken Egg White Derivatives on Chemotactic Activity of Feline Peripheral Blood Polymorphonuclear Cells

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고양이 말초혈액 다형핵백혈구의 유주활성에 있어서 계난백유래물질의 면역증강 효과

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요 약 : 고양이 말초혈액 다형핵백혈구 (PMN)의 유주활성에 있어서 계난백유래물질 (EWD)의 면역증강 효과를 검토하였다. PMN의 유주활성은 EWD 그 자체 및 EWD로 배양한 PMN의 배양상층액에서는 활성이 존재하지 않았다. 그러나 EWD로 배양한 MNC 배양상층액에서는 PMN에 대한 유주활성이 증가되었다. 본 활성은 checkerboard assay를 통해 진성 유주활성으로 밝혀졌다. 이러한 고양이 PMN 유주활성은 *human recombinant interleukin-8 (hr IL-8)*에 의해서도 증가되었다. IL-8의 또 다른 특성인 세포 형태변형 실험에서도 EWD로 배양한 MNC의 배양상층액과 hr IL-8 모두에서 높은 수준의 세포 형태변형효과를 나타내었다. 이와 같이 EWD로 배양한 MNC의 배양상층액과 *hr IL-8* 모두에서 증가된 PMN의 유주활성 및 세포변형효과는 anti-human IL-8 mAb에 의해 농도의존적으로 억제되었다. 이상의 결과로부터 EWD는 고양이 PMN의 유주활성에 있어서 면역증강 효과가 있으며, 이것은 EWD에 의해 활성화된 MNC에서 분비되는 *IL-8 様* 유주성 인자에 의해 PMN의 유주활성 증가 및 세포 형태변형이 일어나는 것으로 사료되었다.

Key words : cat, chemotactic activity, egg white derivatives, polymorphonuclear cells

Introduction

In the recent years, opportunistic infections primary attributable to stress-associated immunosuppression and *complicated infections* difficult to treat with antibiotics alone have been issued in the field of animal clinic. In particular, infectious diarrhea and respiratory diseases, which occur frequently in young companion and domestic food animals, can be prevented only in part by specific immunity with vaccines because multiple pathogenic bacteria contribute to the diseases. Therefore, there is a need for an immunostimulator as an effective means to enhance nonspecific host defence mechanisms against infections.

In immune system, mononuclear cells (MNC) and polymorphonuclear cells (PMN) play major roles in specific or nonspecific immunity. PMN have many functions such as adhension, movement, phagocytosis, degranulation and oxidative burst[1,24]. The passage of PMN from blood vessel into inflammatory sites involves a number of discrete steps including rolling, adhesion and transmigration[5,16]. Transmigration of PMN is also known to be mediated by several chemoattractants such as fMLP (n-formyl-methionine-leucine-phenylalanine) of bacterial origin[22,25], complement-derived C5a[7,11], leukotriene B_4[14], and IL-8[26], etc. *IL-8 is an inflammatory cytokine which plays an* active role in immune reaction[20,21]. It is a monocyte/macrophage-derived peptide that belongs to a novel cytokine family of 6 to 8 kd molecular mass[13] and stimulates neutrophils to directed migration[6].

Chicken egg white derivatives (EWD) originated

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from chicken egg white were mainly composed of ovoalbumin (54%), conalbumin (12%) and ovomucoid (11%). The minor components were composed of ovoinhibitor (1.5%), flavoprotein (0.8%), ficin-papain inhibitor (0.05%), ovomucin (3.5%), ovoglycoprotein (1.0%), ovomacroglobulin (0.5%), ovoglobulin G_2 (4%) and ovoglobulin G_3 (4%)[9]. All of EWD, active egg white product (AEWP) and chicken egg white-derivative immunoactive peptide (EF 203), which are supposed to stimulate macrophage- and neutrophil-functions, have also been demonstrated to enhance nonspecific immunity in mice, piglets, cattle[10], and rainbow trouts[2,17]. The previous studies[12,27-29] suggested that *in vitro* and *in vivo* treatment of EWD might enhance the phagocytosis of peripheral blood phagocytes of cats and rats. Therefore, the aim of this study is to examine the immunostimulating effects of EWD on chemotactic activity of feline PMN.

Materials and Methods

Animals

Healthy cats of average one and half years old were housed at animal cage. All cats were kept at room temperature and 12 hours light cycle and fed on a pellet diet (Fildmaster, Purina Korea, Seoul, Korea) and tap water.

Reagents

The EWD was kindly provided by Eisai Co., Ltd., Tokyo, Japan. EWD was passed through a 0.45 μm-membrane filter before use. Human recombinant (hr) IL-8 and monoclonal antibody (mAb) against human IL-8, IgG_1, were commercially purchased (Sigma, MO, USA).

MNC and PMN isolation

Blood was collected in heparinized tube by jugular venipuncture. Blood diluted 1:1 in phosphate-buffered saline (PBS) at pH 7.6 was layed on the equal volume of Lymphoprep (specific gravity, 1.077; Nycomed Pharma As, Oslo, Norway) and centrifuged at 400×g for 40 minutes at room temperature. MNC in interface between PBS plus plasma and Ficoll-hypaque solution was obtained and subjected to 0.83% NH_4Cl in Tris-base buffer (pH 7.6) containing 1% bovine serum albumin (BSA; Sigma) for 5 minutes at 37°C. One ml of pellet of erythrocytes sediment after removal of MNC layer was mixed with 10 ml of 1.5% dextran (molecular weight, 200,000; Wako, Osaka, Japan) in PBS and allowed to sediment for 60 minutes. The floating PMN of upper compartment were collected and centrifuged at 400×g for 5 minutes. All cells were resuspended in RPMI 1640 (Sigma) supplemented with 2 mM L-glutamine, 0.02 mg/ml of gentamicin, and 5% fetal bovine serum (FBS; Gibco, NY, USA).

Culture supernatant

The PMN and MNC at a density of 2×10^6 cells/ml in a well of a 24-well tissue culture plate (Falcon 3047, Becton Dickinson Labware, NJ, USA) were incubated with concentration of 200 μg/ml of EWD for 24 hours at 37°C under 5% CO_2-humidified atmosphere, respectively. The supernatant were collected by centrifugation (5,000×g for 30 minutes) and stored at −70°C until use for assay.

Chemotaxis assay

Chemotaxis was measured by a modified Boyden chamber assay[7]. The chemotaxis chamber (Neuro Probe, MD, USA) and RPMI 1640 medium containing 1% bovine serum albumin (BSA) were prewarmed for 2 hours at 37°C before use. Nitrocellulose filters were placed on the top of the lower chamber filled with 200 μl of chemoattractant samples. Culture supernatant incubated without EWD referred thereafter to "medium alone" was added in the lower compartment as control. Then, 200 μl of PMN suspension (2×10^6 cells/ml) was placed in the upper compartment. The chambers were incubated for 45 minutes at 37°C in 5% CO_2-humidified atmosphere. After incubation, the membrane filters were immediately taken out and stained with hematoxylin. The migrated distance of PMN through the filter toward the other side was measured by microscopy at 400× magnification. The chemotactic responsiveness of input PMN were evaluated as absolute distance (μm/45 minutes) in the directional migration of PMN in response to chemoattractant.

Checkerboard assay

Checkerboard assay was carried out according to the method of Zigmond and Hirsh[30].

Shape changes of PMN

Morphological changes of PMN were performed as described previously[23]. The mAb against human IL-8 was incubated with culture supernatant from feline MNC treated with EWD and hr IL-8 for 30 minutes at room temperature, respectively. Fifty μl of PMN suspension (2×10^6 cells/ml) were exposed to mixed samples for 30 minutes at 37°C and then fixed in 1.0% glutaraldehyde (Amresco, OH, USA). Fixed cells were stained with Wright-Giemsa stain solution. The percentages of cells showing spherical, ruffled, bipolared, and uropod shape were then determined by a microscope (×400).

Data analysis

The Student's *t* test was used for statistical significance determinations. All data expressed mean ± standard error (SEM).

Results

Effect of EWD and culture supernatants *from PMN and MNC treated with EWD on* feline chemotaxis

As shown in Fig 1, feline PMN in response to EWD was not migrated when compared with control cells. PMN did not also show any migration in response to culture supernatant from PMN treated with EWD (Fig 2). However, culture supernatant from MNC treated with EWD showed a *significant enhancement* ($p<0.01$) *of PMN chemotaxis* as compared with control cells. This enhancement peaked at 1/16 and 1/4 dilution of culture supernatant (Fig 3)

Checkerboard assay

To determine whether PMN migration is a true chemotaxis, the checkerboard assay was performed. *As shown in Table 1, PMN migration strongly* depended on both the increase of a concentration gradient of culture supernatant in lower chamber

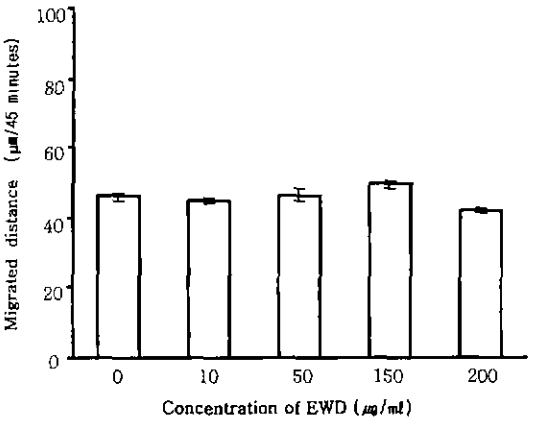

Fig 1. Chemotactic response of feline PMN to EWD. The values represent mean±SEM, n=3.

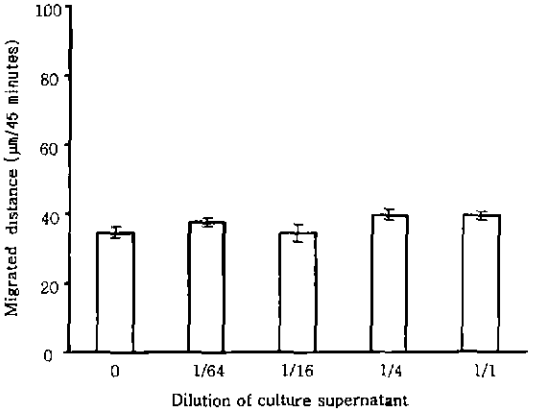

Fig 2. Chemotactic response of PMN to culture supernatant from PMN (2×10^6 cells/ml) treated with EWD (200 μg/ml) for 24 hr. The values represent mean±SEM, n=3.

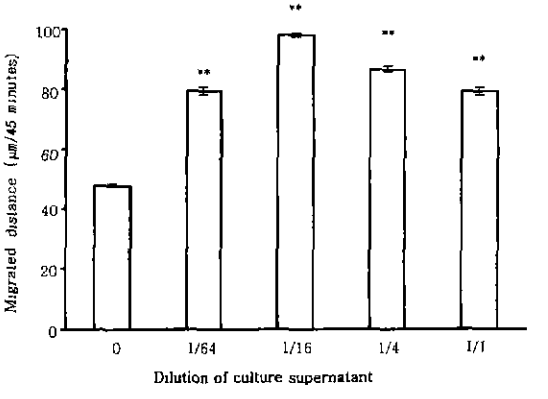

Fig 3. Chemotactic response of PMN to culture supernatant from MNC (2×10^5 cells/ml) treated with EWD. The values represent mean±SEM, n=3. **p<0.01, compared with control (medium alone).

Table 1. Checkerboard assay of feline PMN migration to culture supernatant from MNC treated with EWD

Dilution of culture supernatant in upper chamber	Dilution of culture supernatant in lower chamber				
	0	1/64	1/16	1/4	1
0	42.3±0.9	47.6±0.8	56.6±0.6	66.3±1.2	69.0±0.9
1/64	39.0±0.8	41.6±0.5	45.6±0.8	57.3±0.3	67.6±1 2
1/16	34.0±1.5	39.0±0.6	42.3±0.9	48.3±1.8	66.3±2.1
1/4	28 6±1.8	33.3±0.9	37.3±2.8	44.3±3.0	56.3±2.1
1	26.0±1.2	32.0±2.8	36.2±3.0	39.0±3.5	52.0±3.3

Values of migrated distance (μm/45 minutes) represent as mean±SEM of three determination.

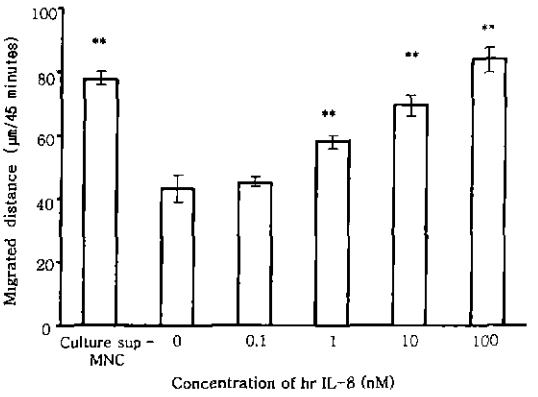

Fig 4. Chemotactic response of feline PMN to hr IL-8 and culture supernatant (1/16) from MNC treated with EWD. The values represent mean±SEM, n=3. **p<0.01, compared with control (medium alone).

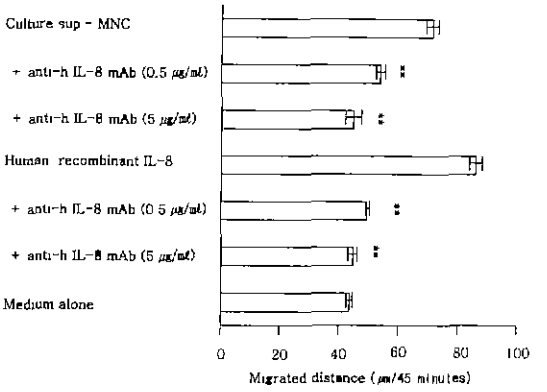

Fig 5. Effect of mAb against human IL-8 on feline PMN chemotaxis by either culture supernatant (1/16) from MNC treated with EWD or hr IL-8 (10 nM). The values represent mean±SEM, n=3. **p<0.01, compared with culture supernatant (1/16) from MNC treated with EWD and hr IL-8 (10 nM), respectivly.

and the decrease of a concentration gradient in upper chamber. This indicated that chemotactic activity of PMN by culture supernatant from MNC treated with EWD was ture chemotaxis but not random migration.

Effect of hr IL-8 on feline PMN chemotaxis

The chemotactic activity of feline PMN to hr IL-8 was also evaluated. As shown in Fig 4, hr IL-8 enhanced the chemotactic activity of feline PMN at concentrations of 0.1 to 100 nM in a dose-dependent manner ($p<0.01$).

Neutralization effect of anti-human IL-8 mAb on chemotactic activity

Neutralization effect of mAb against human IL-8 on the enhanced PMN chemotaxis in response to either culture supernatant from MNC treated with EWD or hr IL-8 was examined. As shown in Fig 5, the chemotactic activity of PMN in culture supernatant from MNC treated with EWD was inhibited ($p<0.01$) in a dose-dependent manner by addition of mAb against human IL-8 at concentrations of 0.5 to 5 μg/ml. Similarly, the inhibitory effect of mAb against human IL-8 was also observed in the chemotactic activity of feline PMN to hr IL-8.

Shape changes of feline PMN

To examine the another characteristics of IL-8, shape change responses of feline PMN to either culture supernatant from MNC treated with EWD or hr IL-8 were tested (Fig 6). Feline PMN receiving EWD (200 μg/ml) alone appeared to be round shape as observed in control cells treated with medium alone. But both culture supernatant (1/16) from MNC treated with EWD and hr IL-8 (10 nM) induced

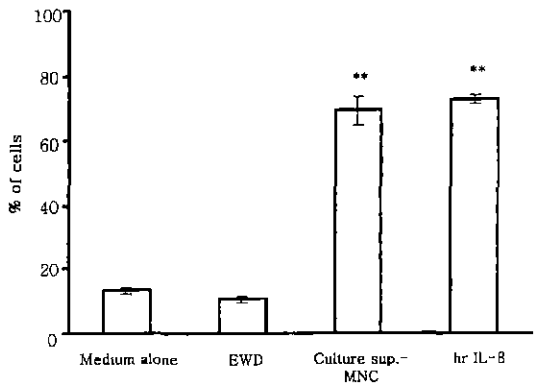

Fig 6. Shape change of feline PMN in treated with EWD (200 μg/ml), culture supernatant (1/16) from MNC treated with EWD and hr IL-8 (10 nM). The values represent mean±SEM, n=3 **p<0.01, compared with medium alone.

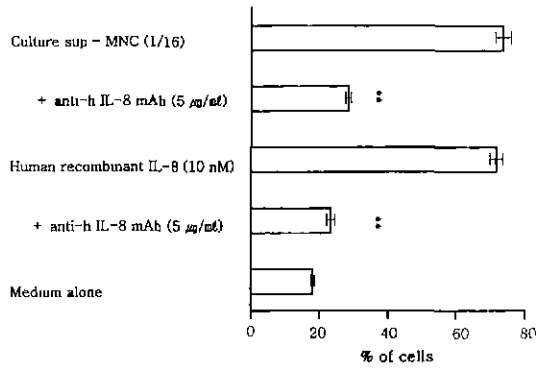

Fig 7. Effect of mAb against human IL-8 on shape change of feline PMN. The values represent mean±SEM, n=3. **p<0.01, compared with culture supernatant (1/16) from MNC treated with EWD and hr IL-8 (10 nM), respectively.

strong and rapid shape change responses ($p<0.01$) of feline PMN as compared with control. Within 30 minutes, the shape of PMN changed in bipolar and spherical form. Addition of mAb against human IL-8 (5 μg/ml) showed a significant inhibitory effect ($p<0.01$) on the shape change responses of feline PMN induced by either culture supernatant from MNC treated with EWD or hr IL-8, respectively (Fig 7).

Discussion

The present results showed that EWD, composed of several proteins of chicken egg white, was not effective on the migration of feline PMN. It is thought that EWD itself may be less responsible for feline PMN chemotaxis. Culture supernatant from PMN treated with EWD was also not chemotactic for PMN. This results indicated that PMN did not release chemotactic factor(s) by EWD stimulation. This finding was consistent with that of chemotaxis for canine PMN[10]. However, culture supernatant from MNC treated with EWD highly enhanced chemotactic activity for PMN. This results may support that most enhancing effect of EWD on chemotactic response of feline peripheral blood PMN was mediated by soluble products produced by EWD-activated MNC but not PMN.

Our results also demonstrated that this migration of PMN to culture supernatant from MNC treated with EWD was true chemotaxis by concentration gradients of chemoattractants rather than random migration, called chemokinesis. Therefore, it is suggested that EWD was capable of releasing the chemoattractants from MNC consisting of monocytes and lymphocytes and that EWD is able to stimulate the nonspecific immune response in feline PMN chemotaxis. Previous results[9,15] showed that ovomucoid, conalbumin, ovoinhibitor, flavoprotein, and ficin-papain inhibitor, components of EWD, induced the remarkable production of chemotactic and phagocytic factors by canine MNC, whereas ovoalbumin, a major component of EWD, did not induce the enhanced chemotactic and phagocytic responses of PMN. Therefore it is of great interest that the magnitude in the enhancement of chemotaxis and phagocytosis of PMN is dependent on the different components of EWD, suggesting the involvement of different active soluble products in the enhancement of chemotaxis and phagocytosis of PMN.

Feline PMN chemotaxis was also induced by human IL-8. Both culture supernatant from MNC treated with EWD and human IL-8 also induced strong and rapid shape changes of feline PMN. Chemotaxin-induced shape changes involve restructuring of cytoskeletal elements modulated in part by alteration of actin polymerization in the cells[4,31]. Dog neutrophils have been found in bipolar shapes when

activated by hr IL-8[23]. Our results suggested that feline IL-8-like chemotactic factor(s) exist in culture supernatant from MNC treated with EWD. The cellular responses mediated by IL-8 were transmitted via specific receptors[19]. High affinity receptors for IL-8 (67 kd and 59 kd) were identified on the surface of human neutrophils that bind IL-8 but not IL-1 and tumor necrosis factor-α (TNF-α)[3,18].

PMN chemotactic responses to hr IL-8 were examined in various animal species[25,31]. Neutrophil migrations of humans, monkeys, hamsters, and dogs to hr IL-8 exhibited increased chemotactic activities, but those of rabbits, rats, and mice showed a low responsiveness to hr IL-8. Chemotactic activities and shape change responses of feline PMN induced by either culture supernatant from MNC treated with EWD or hr IL-8 were inhibited by mAb against hr IL-8. These results suggested that feline IL-8-like chemotactic factor(s) were recognized by mAb against human IL-8. This fact was in part agreement with the reports that chemotactic activities by culture supernatant from bovine MNC treated with LPS were inhibited by ascites containing anti-human IL-8 antibody[8].

In summary, the present study suggested that EWD activates MNC to release IL-8-like chemotactic factor(s), which may be an important mechanism for the enhancement of chemotactic activity of feline PMN. The elucidation of chemotactic factor(s) produced from feline MNC treated with EWD will be considerably important data in the study of immunostimulators

Conclusion

The immunostimulating effect of EWD on chemotactic activity of feline PMN was exaimned. EWD itself and culture supernatant from PMN treated with EWD were not active chemotactic to PMN. But culture supernatant from MNC treated with EWD enhanced the chemotactic activity of feline PMN. The migration of PMN by culture supernatant of EWD-treated MNC was found to be true chemotaxis by checkerboard assay. This chemotactic activity was also induced by hr IL-8. Both culture supernatant of EWD-treated MNC and hr IL-8 also induced strong shape change of feline PMN. In addition, chemotactic activity and shape change of PMN promoted by either culture supernatant of EWD-treated MNC or hr IL-8 were inhibited in a dose-dependent manner by addition of mAb against human IL-8. This study suggested that feline MNC treated with EWD releases feline IL-8-like chemotactic factor(s), which induce the chemotaxis and shape change response of PMN.

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