

Enhancing Effects of Chicken Egg White Derivatives on the Phagocytic Response in the Dog

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ABSTRACT. Immunostimulative effects of chicken egg white derivatives (EWD) on phagocytic responses of peripheral blood mononuclear cells (MNC) and polymorphonuclear cells (PMN) in dogs were evaluated by flow cytometric analysis. Peripheral blood leukocytes (PBL) cultured with EWD showed the enhanced phagocytic response. The response was maximal when PBL were cultured with 100–400 µg/ml of EWD for 3–12 hr. Furthermore, significantly increased phagocytic responses were also induced even when PBL were cultured with protein components (200 µg/ml) of EWD such as conalbumin, flavoprotein and ficin-papain inhibitor for 3 hr. In addition, the enhancing effect of EWD on the phagocytic responses was also observed in MNC cultured with EWD (200 µg/ml) for 4 hr but not in PMN cultured with EWD in the same procedures. The supplement of the supernatant (20%) of MNC cultured with EWD (200 µg/ml) for 24 hr at 37°C to PBL and MNC resulted in the enhancement of their phagocytic responses. In contrast, the supernatant of PMN cultured with EWD for 24 hr at 37°C did not show any significant enhancing effect on the phagocytic responses of PBL, MNC and PMN. These results suggest that EWD has an enhancing effect on phagocytosis of MNC and PMN, which may be mediated through active humoral substances produced by EWD-stimulated MNC.—**KEY WORDS:** canine, egg white derivative, mononuclear cell, phagocytosis, polymorphonuclear cell.

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Immunomodulators are defined as agents which have immunomodulating activity. They include a number of cytokines, biological response-modifiers (BRM) which are involved in the modification of biological responses such as microorganisms or other bacterial components including lipopolysaccharide and muramyl dipeptides, and levamisole, lysostaphin, tuftsin, azimexon, and thymic hormone [10, 11]. A variety of native and synthesized BRM have been developed for disease control. Almost all of these BRM have been reported to have immunomodulating effects on the host in regard to resistance to infectious organisms [2–9, 14–16, 18, 22, 25, 32, 34, 35].

Chicken egg white derivatives (EWD), an active egg white product (AEWP), and chicken egg white-derived immunoactive peptides (EF 203), which are supposed to stimulate macrophage- and neutrophil-functions, have also been demonstrated to enhance nonspecific immunity in mice [3], piglets [4], cattle [22] and rainbow trout [33]. There is, however, little information concerning the action mechanisms and active components of EWD in immunopotentiating effects.

In the present study, therefore, the effects of EWD and its components on the phagocytic responses of mononuclear cells (MNC) and polymorphonuclear cells (PMN) in the peripheral blood of dogs were evaluated by flow cytometry, and a possible involvement of EWD in the enhancement of the phagocytic responses of MNC and PMN is described.

MATERIALS AND METHODS

Animals: Four clinically healthy beagles (2 males and 2 females; 2–4 years old), maintained at the National Institute

of Animal Health, Ministry of Agriculture, Forestry and Fisheries of Japan, were used. They were kept at conventional animal room with commercial diet (DS; Oriental Yeast Ltd., Tokyo) and water *ad libitum*.

EWD: The EWD product was kindly provided by Eisai Co., Ltd., Tokyo, Japan. After the fermentation of fresh egg white samples with *Saccharomyces cerevisiae*, saccharose, lysozyme and avidin were removed by cation column chromatography. The powdered substance dried by spraying at 56°C, referred to as EWD, was dissolved with phosphate buffered saline (PBS; 0.01 M, pH 7.2) to a concentration of 1 mg/ml. The components of EWD, purified by column chromatography and then analyzed for their molecular weights and isoelectric points by electrophoresis, were also kindly provided by Eisai Co.,

Table 1. The composition of EWD

Protein components	Composition rate (%)	Isoelectric points	Molecular weights
Ovalbumin	54.0	4.5	46,000
Conalbumin	12.0	6.05	76,600
Ovomucoid	11.0	4.05	58,000
Ovoinhibitor	1.5	5.1	49,000
Flavoprotein	0.8	4.0	32,000
Ficin-papain inhibitor	0.05	5.1	12,700
Ovomucin	3.5	45, 5.0	— ^{a)}
Ovoglycoprotein	1.0	3.9	24,400
Ovomacroglobulin	0.5	4.5	76,000, 90,000
Ovoglobulin G ₂	4.0	5.5	—
Ovoglobulin G ₃	4.0	5.8	—

a) Not tested.

Ltd. The composition of EWD used in the present study is summarized in Table 1. EWD was consisted of main components of ovoalbumin (54%), conalbumin (12%) and ovomucoid (11%), and minor components of ovoinhibitor (1.5%), flavoprotein (0.8%), ficin-papain inhibitor (0.05%), ovomucin (3.5%), ovoglycoprotein (1.0%), ovomacroglobulin (0.5%), ovoglobulin G₂ (4%) and ovoglobulin G₃ (4%). EWD and each component were then passed through a 0.45 μ m-membrane filter (Millipore Continental Water Systems, Bedford, MA, U.S.A.) before used. The concentration of lipopolysaccharide in the EWD solution was less than 0.05 ng/ml when determined by limulus test [26].

Preparation of peripheral blood leukocytes (PBL), MNC and PMN of dogs: Peripheral blood, taken from the jugular vein of dogs, was collected in a heparinized tube. For the preparation of PBL, the blood samples were centrifuged at 200 g for 30 min at room temperature. The buffy coat was collected, washed three times with PBS, and then supplemented with 0.83% NH₄Cl in Tris-HCl buffer (pH 7.6) containing 1% bovine serum albumin (0.83% NH₄Cl-Tris-HCl) for 10 min at 37°C to remove contaminated red blood cells. The resulting cells were suspended to the desired concentration in complete medium (CM) which was medium RPMI1640 (Nissui Pharmaceutical Co., Tokyo, Japan) containing L-glutamine (0.3 mg/ml), penicillin (100 units/ml), streptomycin (100 μ g/ml) and 10% fetal calf serum, and used as PBL.

For the preparation of MNC and PMN, two fold diluted blood samples in PBS were layered on the equal volume of Lymphoprep (specific gravity 1.077, Nycomed Pharma AS, Oslo, Norway), and centrifuged at 200 g for 30 min. The cells collected from the interface between PBS plus plasma and Lymphoprep were exposed to 0.83% NH₄Cl-Tris-HCl for 10 min at 37°C to remove contaminated red blood cells. The cells were washed three times with PBS. The resulting cells were suspended to the desired concentration in CM, and used as MNC [12, 13, 21].

After the removal of the MNC layer, a layer of red blood cells was collected, and suspended with RPMI1640 medium containing 1.5% dextran (m.w., 20,800, Nacalai Tesque Inc., Kyoto, Japan). Subsequently, the cells were allowed to stand for harvesting floating cells during the sedimentation of red blood cells, and then removed contaminated red blood cells in the same procedure as described above. After washing three times with CM, the resulting cells were suspended to the desired concentration in CM, and used as PMN [13, 21].

The purities of the MNC and PMN used were nearly 85 and 95%, respectively, when determined in the smears stained with Giemsa stain. The viability of the MNC and PMN was always over 95%, when determined by trypan blue (0.25%) exclusion test [13].

Preparation of culture supernatants from EWD-stimulated MNC and PMN: The culture supernatants of EWD-stimulated MNC and PMN were prepared as described previously [13, 21]. In brief, MNC and PMN (2×10^6 cells/ml) were incubated with EWD at a concentration of 200 μ g/

ml for 24 hr at 37°C in a 5% CO₂-humidified air atmosphere. All culture supernatants were collected by centrifugation (5,000 g, for 30 min), and then filtered with 0.45 μ m-Millipore filters.

Phagocytosis assay: The phagocytic activity of peripheral blood phagocytes was determined as described previously [11]. Five hundreds μ l of PBL, MNC and PMN (2×10^6 cells/ml) were incubated in a well of a 24-well plate (Falcon, Becton-Dickinson and Co., NJ, U.S.A.) with EWD at the different concentrations ranging from 12.5 to 400 μ g/ml, each component (200 μ g/ml) of EWD or each culture supernatant (20%) of EWD-stimulated MNC or PMN for 3 hr at 37°C, in a 5% CO₂-humidified air atmosphere. Subsequently, the cells were supplemented with 1×10^9 particles of FITC-conjugated Latex beads (Latex beads; 2.0 μ m; Polysciences Inc., Warrington, U.K.), and then incubated for another 1 hr. The cells cultured with CM or Latex beads alone were used as negative controls. The cells were washed three times and immediately analyzed by a flow cytometer (EPICS-Profile II, Coulter Inc., Hialeah, FL, U.S.A.). The phagocytosis assay was done in triplicate. The experiments concerning the effects of the supernatants from EWD-stimulated MNC or PMN on phagocytosis of freshly prepared PBL, MNC and PMN was repeated three times. Positive staining was determined by using "Overtons Cumulative Subtraction" method which is a one-parameter histogram subtraction routine for test histograms with weak immunofluorescence. The results were expressed as the mean of gross or net increased relative proportion of phagocytosed cells in a monocyte-rich fraction in MNC and a neutrophil-rich fraction in PMN, which were calculated on the basis of 2 or 3 repeated experiments, as described previously [13].

Statistics: Student *t*-test was used in the comparison of means. Estimate of variance, where shown, is 1 standard error (SE).

RESULTS

Kinetics of the phagocytic response of PBL cultured with EWD: The kinetics of the phagocytic response in EWD-stimulated PBL are shown in Fig. 1. The net increased relative phagocytic activity reached a maximal level, when PBL were stimulated with EWD at the concentrations ranging from 100 to 400 μ g/ml for 3 to 12 hr. On the other hand, the phagocytic activity of PBL cultured for 24 hr with 100–400 μ g/ml of EWD was less than approximately a half of the maximal level.

Effects of the components of EWD on the phagocytic response of PBL: Phagocytic activities of PBL cultured with each component (200 μ g/ml) of EWD, ovoalbumin, conalbumin, ovomucoid, ovoinhibitor, flavoprotein and ficin-papain inhibitor for 3 hr were evaluated. As shown in Fig. 2, PBL stimulated with conalbumin, flavoprotein, and ficin-papain inhibitor exhibited a significantly higher phagocytic activity which was within the level comparable to that of PBL stimulated with EWD ($45.2 \pm 6.2\%$). On the other hand, ovoalbumin, ovomucoid and ovoinhibitor did

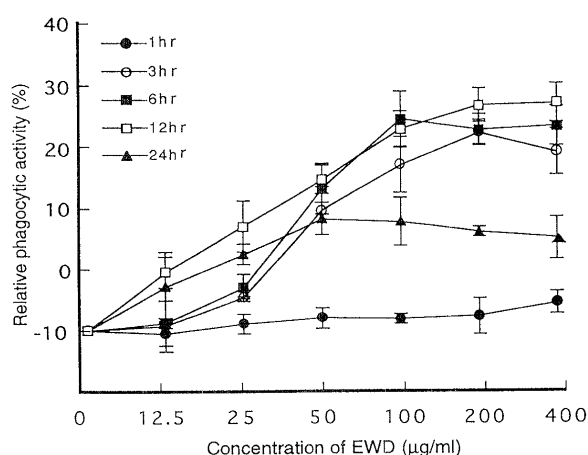


Fig. 1. Kinetics of relative phagocytic activities of canine PBL cultured with EWD. PBL (2×10^6 cells/ml) were incubated with EWD (200 μ g/ml) for 1, 3, 6, 12 and 24 hr and their relative phagocytic activities were analyzed by flow cytometry. The value represents the mean \pm SE in the triplicate assay.

Table 2. Effects of EWD and culture supernatants of EWD-stimulated MNC and EWD-stimulated PMN on phagocytic activities of PBL, MNC and PMN

Cells	Supplement with	Phagocytic activity (%)
PBL	medium	13.5 \pm 3.5
	EWD	35.7 \pm 4.3*
	culture supernatant	29.7 \pm 4.3*
	from EWD-stimulated MNC	
MNC	culture supernatant	11.5 \pm 3.5
	from EWD-stimulated PMN	
	medium	27.6 \pm 2.2
	EWD	45.7 \pm 4.2*
PMN	culture supernatant	45.5 \pm 3.2*
	from EWD-stimulated MNC	
	culture supernatant	30.4 \pm 5.3
	from EWD-stimulated PMN	
PMN	medium	3.4 \pm 1.2
	EWD	4.3 \pm 2.4
	culture supernatant	36.5 \pm 2.5*
	from EWD-stimulated MNC	
	culture supernatant	7.4 \pm 3.2
	from EWD-stimulated PMN	

* $P < 0.01$, when compared to medium control.

not induce a significantly enhanced phagocytic response of PBL.

Effects of EWD and the culture supernatants of EWD-stimulated MNC and PMN on phagocytosis of PBL, MNC and PMN: The results of three experiments concerning the phagocytic activities in PBL, MNC and PMN cultured alone or with the culture supernatants from EWD-stimulated MNC and EWD-stimulated PMN for 3 hr are shown in Table 2.

Significantly high phagocytic activities ($35.7 \pm 4.3\%$) were induced in PBL cultured with EWD (200 μ g/ml), when compared to those ($13.5 \pm 3.5\%$) in PBL cultured without EWD. The supplement of the culture supernatant (20%)

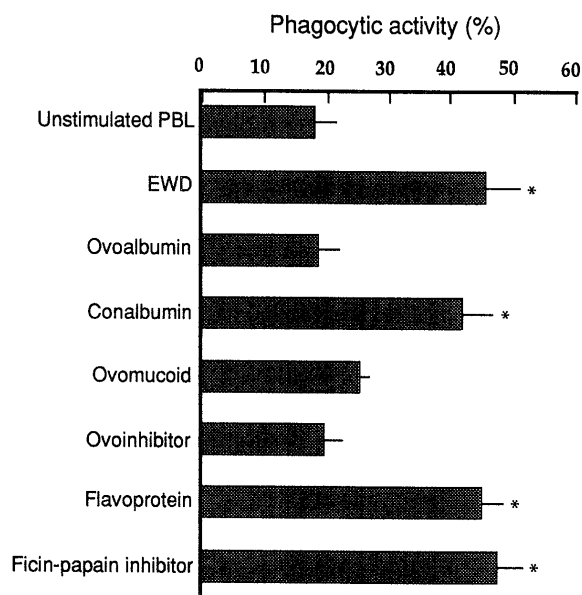


Fig. 2. Phagocytic activities of canine PBL cultured with each component of EWD. The value represents the mean \pm SE in the triplicate assay. * $P < 0.01$, when compared to unstimulated PBL.

from EWD-stimulated MNC to PBL also induced significantly enhanced phagocytosis ($29.7 \pm 4.5\%$) whereas the culture supernatant of EWD-stimulated PMN did not show such an enhancement of phagocytosis of PBL.

MNC cultured with EWD (200 μ g/ml) showed significantly increased phagocytosis ($45.7 \pm 4.2\%$), when compared to MNC cultured without EWD ($27.6 \pm 2.2\%$). The supplement of the culture supernatant from EWD-stimulated MNC or EWD-stimulated PMN to freshly prepared MNC also resulted in the enhancement of their phagocytosis ($45.5 \pm 3.2\%$). On the other hand, the supplement of the culture supernatant from EWD-stimulated PMN to fresh MNC failed to significantly increase their phagocytosis. In addition, the flow cytometric profile of MNC cultured with the supernatant of EWD-stimulated MNC showed that the relative proportion of a phagocytosed cell population in fraction 4 with higher fluorescence intensity markedly increased (data not shown).

Phagocytosis ($4.3 \pm 2.4\%$) of PMN cultured with EWD in the same manner was almost similar to that of unstimulated PMN ($3.4 \pm 1.2\%$). However, the phagocytic activity of PMN significantly increased, when PMN were co-cultured with the culture supernatant from EWD-stimulated MNC ($36.5 \pm 2.5\%$). On the other hand, the supplement of the culture supernatant from EWD-stimulated PMN to freshly prepared PMN did not result in a significant increase in their phagocytosis ($7.4 \pm 3.2\%$).

DISCUSSION

The results concerning the effects of EWD on the *in vitro* phagocytosis of canine peripheral blood phagocytes in the present study are summarized as follows: (1) EWD at a concentration of 200 μ g/ml used here significantly increased

the phagocytic responses of PBL and MNC, whereas PMN cultured in the same manner did not show any marked phagocytic response (Figs. 1 and 2, and Table 2). (2) The culture supernatant of MNC stimulated with EWD for 24 hr also significantly enhanced the phagocytosis of MNC and PMN (Table 2). However, the supernatant of PMN similarly cultured with EWD did not show such significantly enhancing effects on phagocytosis of canine MNC and PMN (Table 2). These results may support that the enhanced phagocytosis of MNC and PMN is substantially induced by humoral factors released from EWD-stimulated MNC but not by EWD remaining in the culture supernatant, since the concentration of EWD (approximately 40 $\mu\text{g}/\text{ml}$) remaining in the culture supernatant of MNC was not effective in the induction of a significantly enhanced phagocytosis of PBL as shown in Fig. 1. As described above, the phagocytic response to EWD was different between neutrophil- and mononuclear cell-rich fractions. It would seem that low phagocytic activities of PMN to EWD were associated with their low responsiveness rather than a decrease in the viability during cultures, since difference in the viability (approximately 50% at 24 hr) between PMN cultured with EWD and PMN cultured without EWD was not observed and supplement of the culture supernatant of EWD-stimulated MNC to PMN resulted in a significantly high phagocytic response. Based on these findings, a possible mechanism in the enhancement of phagocytosis of canine PBL in response to EWD may be assumed as follows. EWD may act on a mononuclear cell population (monocytes and lymphocytes) directly but not on a PMN population to produce active humoral substances which are involved in the enhancement of phagocytosis of MNC and PMN. Therefore, these results suggest that enhancing effects of EWD on the phagocytic response of canine peripheral blood phagocytes are mediated by humoral factors produced by EWD-stimulated MNC (mainly monocytes) but not by EWD-stimulated PMN.

Although humoral factors involving in the enhancement of MNC and PMN functions have not been identified yet, cytokines such as IL-1, IL-2, IL-8, interferons and tumor necrosis factors can be candidates on the basis of the findings that these cytokines have been involved in the modulation of neutrophil functions including adherence [31], migration [17, 20], respiratory burst [24], lysosomal enzyme release, and cell surface receptor expression [1, 9, 27–30].

Recently we have reported that the culture supernatants of EWD-stimulated MNC and lipopolysaccharide-stimulated MNC are chemotactic for canine MNC as well as for PMN, whereas neither EWD nor the culture supernatant of EWD-stimulated PMN are chemotactic for these cells, suggesting that EWD-stimulated PMN have a negligible ability to produce active humoral substances enhancing phagocytosis and chemotaxis of PMN [13, 21]. Furthermore, IL-8 released in the culture supernatant of EWD-stimulated canine MNC but not in that of EWD-stimulated canine PMN has been found to be involved in the enhancement of chemotaxis of PMN and MNC [19, 21]. Our preliminary study also showed that ovomucoid and ovinhibitor,

components of EWD, induced the remarkable production of neutrophil chemotactic factors by canine MNC, whereas they exhibited a negligible production of humoral substances involved in the enhancement of phagocytosis of PBL (data not shown). Therefore, it is of great interest that the magnitude in the enhancement of phagocytosis and chemotaxis of canine PBL is dependent on the different components of EWD used for stimulation of canine MNC and PMN, suggesting the involvement of different active humoral substances in the enhancement of phagocytosis and chemotaxis of canine MNC and PMN. Identification and characterization of humoral factors in the culture supernatant of EWD-stimulated MNC are needed to define the action mechanisms by which the enhancement in phagocyte functions is induced after stimulation with EWD.

Enhancing effects of the products derived from chicken egg whites on nonspecific immunity involving neutrophil- and macrophage-functions have been shown in mice, piglets, cattle, and fish administered AEWP and immunoactive peptides (EF 203). The oral administration of AEWP (1.5 g/kg) resulted in the enhancement of resistance to *E. coli* and *Staphylococcus aureus* infections in mice [3]. Phagocytic activity of neutrophils was significantly enhanced in piglets orally administered AEWP (30 mg/kg/day) [4]. Neutrophils from cattle orally administered AEWP (500–1,000 mg/kg) also showed the enhancement in their functions when evaluated by nitro blue tetrazolium reduction test and intracellular killing of *S. aureus* [22]. Furthermore, the chemiluminescent responses of kidney phagocytes also significantly increased in rainbow trout orally given EF 203 [33]. Therefore, the enhanced nonspecific immunity in these animals administered AEWP or EF 203 would be induced by cytokine-like humoral factors produced by MNC stimulated with EWD-associated products. For clinical applications, more detailed studies including the effects of EWD on immune functions are needed.

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