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Immunoenhancing Effect of Conjugated Linoleic Acids on Phagocytic Activity of Porcine Peripheral Blood Phagocytes

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Abstract : The immunoenhancing effect of CLA isomers (CLA mixture, 10t-12c CLA, 9c-11t CLA, 9c-11c CLA, and 9t-11t CLA) on phagocytic activity of porcine peripheral blood leukocytes was examined. The phagocytic activities of peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMN) were analyzed by a flow cytometry system. The direct treatments of CLA isomers have no effect on phagocytosis of PMN as well as PBMC composed of approximately 10% monocytes and 90% lymphocytes. However, the phagocytic activities of PMN and monocyte-rich fraction from PBMC were remarkably enhanced by culture supernatant from PBMC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA but not 9c-11c CLA and 9t-11t CLA. The phagocytic activity of PBMC was not enhanced by culture supernatant from PBMC treated with all CLA isomers. These results indicated that CLA isomers such as CLA mixture, 10t-12c CLA and 9c-11t CLA have an enhancing effect on phagocytosis of PMN and monocytes, which may be mediated through active humoral substances produced by CLA-stimulated PBMC. This study suggested that CLA stimulates PBMC to elaborate soluble factor(s), which may be an important mechanism for the enhancement of phagocytosis in non-specific immunity.

Key words **:** pig, conjugated linoleic acid, peripheral blood phagocytes, phagocytosis.

Introduction

Conjugated octadecadienoic acids are a group of geometric and positional isomers of linoleic acid (C18:2, n-6). They are collectively known as conjugated linoleic acid (CLA). CLA isomers include both *cis-cis*, *cis-trans* and *trans-trans* geometry with double bonds at 9 and 11, 10 and 12 or 11 and 13, 7 and 9, 12 and 14 or 8 and 10. These isomers occur naturally and are enriched in the tissues of ruminant animals and in dairy products[8].

CLA may act in part by competing with linoleic acid in the biosynthesis of arachidonic acid[7]. Dietary CLA suppressed the production of prostaglandin E_2 (PGE_2) in serum and spleen[30], keratinocyte[18], and bone[17]. The elongated and desaturated metabolites of the 10t-12c CLA isomer competitively inhibited the conversion of arachidonic acid into PGE_2[20]. PGE_2 suppresses T-lymphocyte proliferation and cytotoxic activities of natural killer cells[11,39] and inhibits the production of tumor necrosis factor (TNF)-α[25] and interleukin (IL)-12[35].

On the other hand, dietary CLA has been shown to increase immunoglobulin production in rat spleen lymphocytes[31]. CLA in physiological concentration inhibits proliferation of several human cancer cell lines *in vitro*[26,28,29]. The 9c-11t CLA and 10t-12c CLA in numerous isomers are known to possess biological activity[4]. The 10t-12c CLA alters lymphocyte blastogenesis[6]. Mixtures of CLA isomers (mostly 9c-11t CLA and 10t-12c CLA) have been shown to enhance the immune system and reduce the catabolic effects of immune stimulation[5]. The 9c-11t CLA and 10t-12c CLA isomers may be equally effective in inhibiting carcinogenesis[13]. When CLA was added to porcine lymphocyte culture *in vitro*, it increased mitogen induced lymphocyte blastogenesis, lymphocyte cytotoxic activity and murine macrophage killing ability[19]. Mice fed 0.3% and 0.9% CLA had increased *in vitro* lymphocyte proliferation in response to phytohemagglutinin[36]. IL-2 production in these mice was also stimulated by CLA. Therefore, it is thought that the multiple effects of CLA may be occurred due to the results of several biochemical mechanisms that involve both direct effects of the CLA isomers as well as metabolites of the CLA isomers.

It was reported that rats fed a diet containing 0.5% CLA had enhanced peritoneal macrophage phagocytosis[5]. However, the effects and/or the roles of CLA on the peripheral blood phagocytes have not been well investigated. More recently we reported that CLA isomers such as 10t-12c CLA, 9c-11t CLA and CLA mixture have an enhancing effect on chemotaxis of porcine PMN, which may be mediated through IL-8-like factor produced by CLA-stimulated PBMC[14]. It was also assumed that PBMC activated by CLA isomers release many factors associated with host defense and immune response. These include a collection of cytokines, such as IL-1, TNF-α, IL-6 and IL-8 that promote immune response[24]. Therefore, the immunoenhancing effect of CLA isomers on phagocytic activity of porcine blood peripheral phagocytes was examined, and a possible invol-vement of soluble products in the enhancement of phagocytic responses of monocytes/PMN by culture supernatant from PBMC and PMN treated with CLA isomers was described.

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Materials and methods

Pigs

Clinically healthy crossbred pigs of average 1-year-old were used as blood donors. All pigs were housed in temperature controlled room which was controlled light with an alternating 12 hours light/dark cycle. Pigs were fed on commercial diet and tap water.

Reagents

CLA mixture (Nu-Chek-Prep Corporation, MN, USA), which was mainly composed of 44% 10t-12c CLA and 41% 9c-11t CLA, were commercially purchased. The 10t-12c CLA, 9c-11t CLA, 9c-11c CLA, and 9t-11t CLA (Matreya, Inc., PA, USA) were also purchased. According to manufacturer's description, the purity of all CLA isomers except for CLA mixture was more than 96%.

PBMC and PMN isolation

Peripheral blood drawn in heparinized tube from anterior venae cava was diluted with the equal volume of phosphate-buffered saline (PBS) at pH 7.6 and layered 1:1 on Ficoll-hypaque solution (specific gravity, 1.080; Sigma-Aldrich Co., St. Louis, MO, USA). After centrifugation at 400× g for 40 min at room temperature, the PBMC in interface between PBS plus plasma and Ficoll-hypaque solution was harvested and washed 3 times with PBS. The resulting PBMC was consisted of approximately 90% lymphocytes and 10% monocytes by modified Wright and Giemsa stain. The PMN was obtained from layer of erythrocyte sediment by treatment of 1.5% dextran (moleculr weight, 200,000; Wako Ltd., Osaka, Japan) after removal of PBMC layer. The erythrocytes allowed to sediment with dextran for 60 min. The floating cells were collected and centrifuged at 400× g for 5 min. Purity of neutrophils in final PMN preparation was greater than 96% when determined by cytospin smear and Giemsa stain. Cell viability of PBMC and PMN ,determined by trypan blue dye exclusion methods, always exceeded 98%. All cells were resuspended in RPMI 1640 (Sigma-Aldrich Co.) supplemented with 2 mM L-glutamine, 0.02 mg/ml of gentamicin and 5% fetal bovine serum (Invitrogen Co., Grand Island, NY, USA) and finally adjusted to 2×10^6 cells/ml.

Culture supernatant

The isolated PBMC and PMN at a density of 2×10^6 cells/ml in a well of a 24-multiwell plate (Nunc Co., Naperville, IL, USA) were incubated with a concentration of 20 μM of CLA isomers for 24 h at 37°C under 5% CO_2-humidified atmosphere. The supernatant was collected by centrifugation (5,000× g for 30 min), filtered with 0.45 μm-pore size membrane filter and stored at -70°C until use for assay.

Phagocytosis assay

The phagocytic activity of PBMC and PMN was determined as described previously[10]. The cells at a density of 1×10^6 cells/ml were incubated for 12 h at 37°C in a 5% CO_2-humidified air atmosphere in a well of a 24-multiwell plate with either CLA mixture, 10t-12c CLA, 9c-11t CLA, 9c-11c CLA and 9t-11t CLA at different concentrations ranging from 10 to 20 μM or each culture supernatant of CLA-treated PBMC and PMN with various concentrations. The control cells were treated with the equal volume of dimethyl sulfoxide (DMSO) which was used in the dilution of CLA isomers. The cultures were thereafter supplemented with 20 μl of 1×10^9 particles/ml of FITC-labelled latex (latex bead size, 2.0 μm; Polyscience, Inc., Warrington, PA, USA) for the final 1 h. The cultured cells were harvested gently by slow pipetting, centrifuged at 2,000× g for 3 min, and washed 3 times with PBS containing 3 mM EDTA-2Na. The phagocytized latex cells per total 10,000 cells in PBMC and PMN were immediately estimated by a flow cytometry (FACS Calibur, Becton Dickinson Immunocytometry Systems, CA, USA). To use the monocyte-rich cells in flowcytometric cytography of PBMC, they were fractioned by cell size of PBMC from dot plot profile in flowcytometric cytography, The phagocytized cells per total 5,000 cells in monocyte-rich fraction were measured. Results were expressed as percentage of absolute phagocytic activities.

Data analyses

The Student's *t*-test was used for statistical significance determinations. All data are expressed as mean ± standard error of means (S.E.M.).

Results

Direct effect of CLA isomers on phagocytosis of porcine peripheral blood phagocytes

To examine the direct effect of CLA isomers on phagocytic activity of porcine peripheral blood phagocytes, freshly isolated PMN and PBMC were cultured with CLA mixture, 10t-12c CLA, 9c-11t CLA, 9c-11c CLA and 9t-11t CLA at different concentrations ranging from 10 to 20 μM for 12 h. The phagocytic activities of PMN (Fig 1), PBMC (Fig 2) and

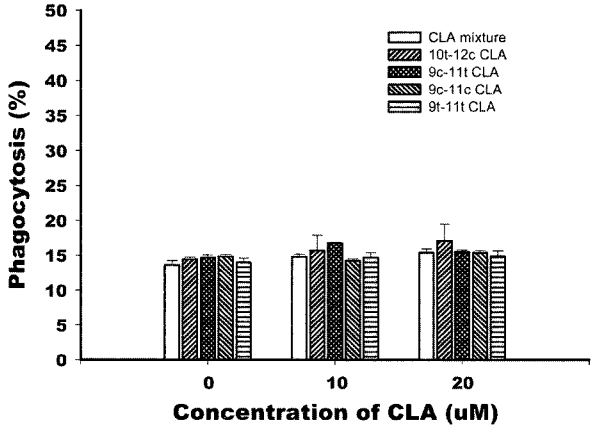

Fig 1. The direct effects of CLA isomers at concentration 10 to 20 μM on porcine PMN phagocytosis. The data represent mean ± S.E.M., (n=3).

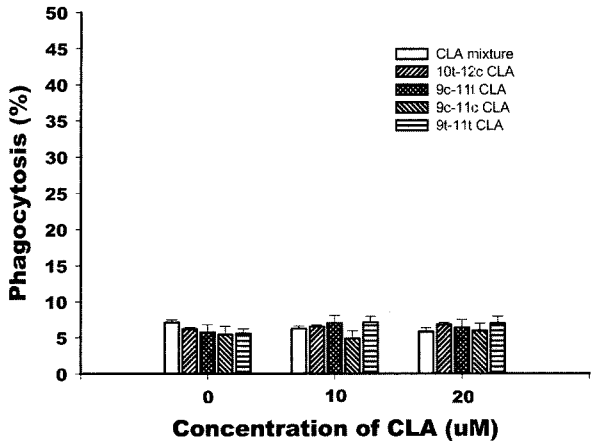

Fig 2. The direct effects of CLA isomers at concentration 10 to 20 μM on porcine PBMC phagocytosis. The data represent mean± S.E.M., (n=3).

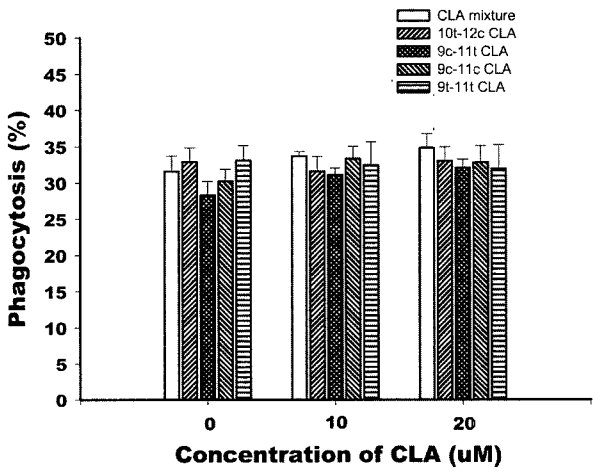

Fig 3. The direct effects of CLA isomers at concentration 10 to 20 μM on porcine monocyte-rich cells fractionated from dot plot profile in flowcytometric cytography of PBMC. The data represent mean± S.E.M., (n=3).

monocyte-rich fraction (Fig 3) were not augmented by each treatment of CLA mixture, 10t-12c CLA, 9c-11t CLA, 9c-11c CLA and 9t-11t CLA as compared with those in cells treated without CLA isomers.

Phagocytic response of porcine PMN by culture supernatant from PBMC treated with CLA isomers

To determine whether phagocytic activity of PMN could be enhanced by addition of culture supernatant from PBMC (2×10^6 cells/ml) treated with CLA isomers (20 μM) for 24 h, freshly prepared PMN was followed by 12 h-incubation with culture supernatant (0 to 75%) from PBMC treated with CLA isomers. The phagocytic activities of PMN were strongly enhanced by addition of culture supernatant from PBMC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA,

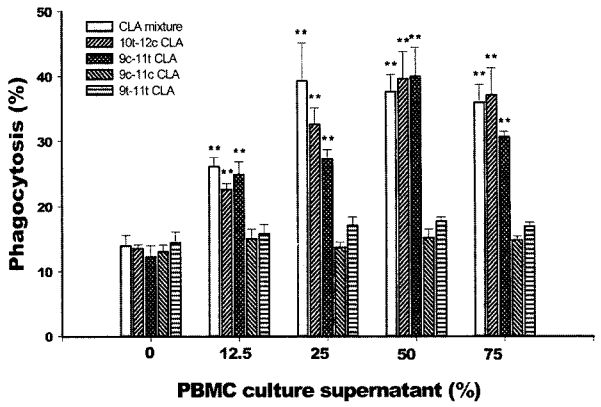

Fig 4. Phagocytic response for porcine PMN by culture supernatant from PBMC treated with CLA isomers (20 μM). The data represent mean± S.E.M., (n=4). $^{**}p<0.01$, compared to control (0%).

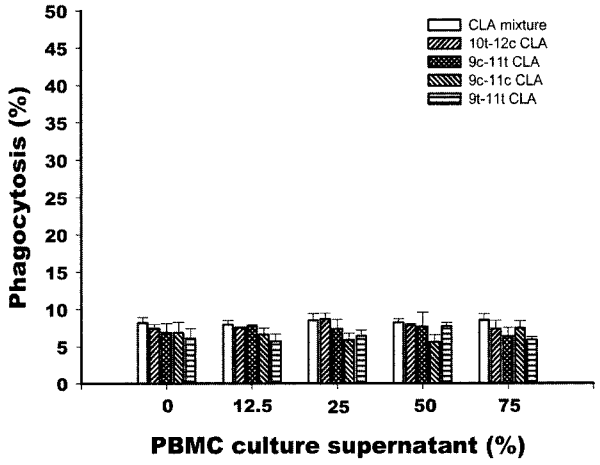

Fig 5. Phagocytic response for porcine PBMC by culture supernatant from PBMC treated with CLA isomers (20 μM). The data represent mean± S.E.M., (n=4).

respectively ($p<0.01$), when compared with those of control cells (Fig 4). These enhancements were a dose-dependent manner. However, the culture supernatant from PBMC treated with 9c-11c CLA and 9t-11t CLA showed no enhancing effect on PMN phagocytosis.

Phagocytic response of porcine PBMC and monocyte-rich fraction by culture supernatant from PBMC treated with CLA isomers

Phagocytic response of porcine PBMC by culture supernatant from PBMC treated with CLA isomers in the same manner was also examined. PBMC did not showed the enhanced phagocytic activity to all CLA isomers (Fig 5). However, monocytes-rich cells fractionated from PBMC in flowcytometric cytography resulted in the enhancement ($p<0.05$ to 0.01) on its phagocytic response by culture supernatant from

PBMC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA when compared with those of control cells (Fig 6). However, its phagocytic activity was not enhanced by the culture supernatant from PBMC treated with 9c-11c CLA and 9t-11t CLA.

Phagocytic response of porcine peripheral blood phagocytes by culture supernatant from PMN treated with CLA isomers

The phagocytic response of phagocytes by culture supernatant from PMN treated with CLA isomers (20 μM) for 24 h was also examined. As a result, the culture supernatant from PMN did not showed any enhancement in phagocytosis of PMN (Fig 7) and monocyte-rich fraction (Fig 8).

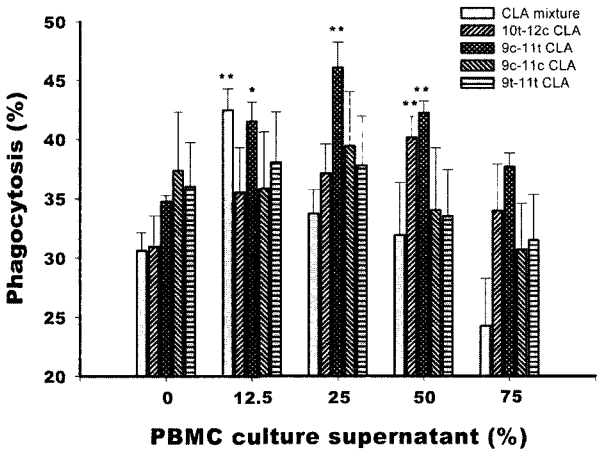

Fig 6. Phagocytic response for porcine monocyte-rich cells by culture supernatant from PBMC treated with CLA isomers (20 μM). The data represent mean±S.E.M., (n=3). *$p<0.05$, **<0.01, compared to control (0%).

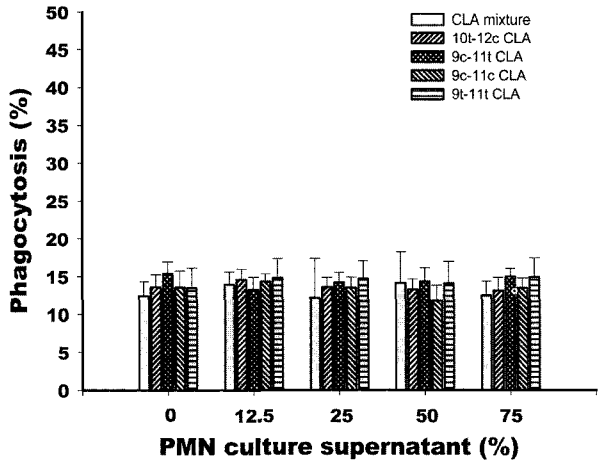

Fig 7. Phagocytic response for porcine PMN by culture supernatant from PMN treated with CLA isomers (20 μM). The data represent mean±S.E.M., (n=3).

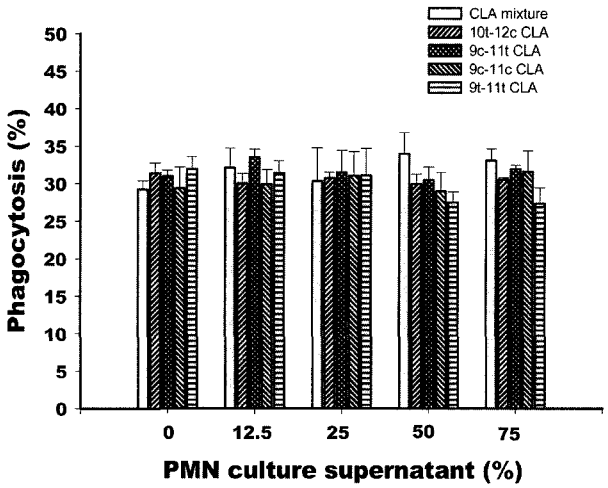

Fig 8. Phagocytic response for porcine monocyte-rich cells by culture supernatant from PMN treated with CLA isomers (20 μM). The data represent mean±S.E.M., (n=3).

Discussion

In the previous study[14], PBMC and PMN at concentraion of 50 to 200 μM of CLA isomers for 24 h-incubation showed the reduced viabilities. It was assumed that CLA isomers at higher concentration show cytotoxicity to peripheral blood leukocytes with a rapid life cycle. Therefore, CLA isomers were used at concentration of 20 μM showing high cell viability and no cytotoxic effect.

The present results revealed that CLA isomers themselves were not effective on the phagocytosis of peripheral blood phagocytes. It has been also suggested that CLA isomers can be incorporated into membrane phospholipids and may replace arachidonic acid. These isomers reduce the release of PGE_2 from antigen-challenged lung, trachea, and bladder in the guinea pig[9,12]. Therefore, it was thought that CLA isomers have no change *in vitro* metabolite production of arachidonic acid in porcine PMN and have directly no effects on phagocytosis of PMN.

The culture supernatant from PBMC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA remarkably enhanced the phagocytic activity for PMN. Whereas culture supernatant from PBMC treated with 9c-11c CLA and 9t-11t CLA failed to induce phagocytic activity for PMN. This was of interest that the presence and absence of phagocytic activity for PMN in culture supernatant from PBMC treated with CLA differs in classes of these isomers. CLA isomers are synthesized by base isomerization of linoleic acid. These have the multiple effects according to biochemical mechanisms. Although it is difficult to explain the reason for presence and absence of phagocytic activity, it is presumed that biological activity of CLA may be also differs in the kinds of its isomers. In fact, only the 9c-11t CLA and 10t-12c CLA are known to possess biological activity[22]. Also, CLA mix-

ture is predominantly consisted of 9c-11t CLA and 10t-12c CLA[21].

The culture supernatant from PBMC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA but not 9c-11c CLA and 9t-11t CLA was capable of enhancing the phagocytic activity of PMN which was consist of approximately 96% neutrophils. The activity of monocyte-rich cells fractionated from PBMC was also enhanced by culture supernatant from PBMC exposed to CLA. However, the phagocytic activity of PBMC by culture supernatant from PBMC treated with CLA isomers was not effective. This may be associated with PBMC population. Since PBMC isolated from peripheral blood was composed of both approximately 10% monocytes and 90% lymphocytes. Therefore, it was suggested that the enhanced phagocytic activity of porcine peripheral blood phagocytes such as neutrophils and monocytes could be mainly mediated by soluble product(s) released from PBMC exposed to CLA isomers. This may support that the soluble products are involved in the enhancement of phagocytosis for phagocytes as paracrine or autocrine manner. Culture supernatant from PMN treated with CLA isomers was not phagocytic for phagocytes, indicating that PMN did not release phagocytic factor(s) by any antigenic or mitogenic stimulation of CLA. These findings were consistent with that of previous studies on phagocytosis for PMN treated with either egg white derivatives (EWD)[10,37,38] or red ginseng saponins[23]. These results strongly suggested that CLA isomers have an immunoenhancing effect on phagocytosis of porcine peripheral blood phagocytes, which may be mediated through active humoral factor(s) produced by CLA-stimulated PBMC.

The soluble products of activated monocyte-macrophages and lymphocytes probably play important roles in the phagocytosis of phagocytes. In addition, PBMC produces many other macrophages- or neutrophil-activating cytokines such as IL-1, IL-2, IL-8, interferons, TNFs and granulocyte-macrophage-colony stimulating factor (GM-CSF), thereby suggesting that it may share some common functions in inflammatory responses[3,33,40]. The ability of PBMC to release the soluble factors and stimulate the phagocytosis of phagocytes may potentially be implicated in the regulation of cellular infiltration at sites of inflammation or tumor growth. It was, therefore, possible that PBMC stimulated with CLA isomers (CLA mixture, 10t-12c CLA and 9c-11t CLA) also modulates the chemotaxis of PMN through the release of humoral factor(s) including IL-8-like factor[14]. The representative phagocytosis-promoting factors produced by activated PBMC are known as IL-1 and TNFs[1,32]. It was suggested that the phagocytosis-promoting factor, which is produced by feline PBMC in response to EWD, one of immunostimulators, is associated with the humoral factor of 16 to 18 kDa[37]. Also, the phagocytic activity for porcine PMN in culture supernatant from PBMC exposed to EWD was identified as TNF-α with molecular weight of 16 to 18 kDa but not IL-1[15]. TNF-α has been shown to be associated with several biological effects including PMN activation and immune regulation[2,16]. It has been reported that TNF-α augmented the phagocytosis and antibody-dependent cytotoxic effector function of polymorphonuclear leukocytes[27,34]. Therefore, additional research is required to determine these active soluble product(s) released by CLA-stimulated PBMC.

The overall results of this study suggested that CLA stimulates PBMC to elaborate soluble product(s), which may be an important mechanism for the enhancement of phagocytosis of porcine peripheral blood phagocytes.

Conclusion

CLA isomers have directly no enhancing effects on the phagocytic activity of PMN and PBMC. Culture supernatant from PBMC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA except for 9c-11c CLA and 9t-11t CLA remarkably enhanced the phagocytosis of PMN and monocyte-rich fraction but not PBMC. However, the culture supernatant from PMN treated with CLA isomers showed no enhancing effect on phagocytosis of PMN and monocyte-rich cells. These results strongly suggested that CLA isomers (CLA mixture, 10t-12c CLA and 9c-11t CLA) have an enhancing effect on phagocytosis of PMN and monocytes, which may be mediated through active humoral factor(s) produced by CLA-stimulated PBMC.

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돼지 말초혈액 탐식세포의 탐식활성에 있어서 CLA의 면역증강효과

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요 약 : 돼지 말초혈액 탐식세포(PMN 및 monocyte)의 탐식성에 있어서 CLA 이성체의 탐식증강 효과를 검토하였다. 탐식세포의 탐식성은 flow cytometry로 분석하였으며 결과는 다음과 같다. 1. CLA 이성체(CLA mixture, 10t-12c CLA, 9c-11t CLA, 9c-11c CLA, 9t-11t CLA)를 직접 첨가하여 배양한 PMN과 PBMC 및 flow cytometry cytography에서 세포크기에 의해 분획한 monocyte-rich fraction에는 탐식증강 효과가 없었다. 2. 각각의 CLA 이성체로 배양한 PBMC 배양상층액으로 PBMC의 탐식활성을 측정한 결과 증강효과는 관찰되지 않았다. 3. CLA 이성체로 배양한 PBMC 배양상층액 중 CLA mixture, 10t-12c CLA, 9c-11t CLA 처리군에서는 PMN과 monocyte-rich fraction의 탐식활성에 있어서 현저한 증강효과가 관찰되었다. 그러나, 말초혈액 탐식세포들은 9c-11c CLA와 9t-11t CLA로 배양한 PBMC 배양상층액에 의해서는 증강효과가 나타나지 않았다. 4. CLA 이성체로 배양한 PMN 배양상층액에 있어서는 PMN과 monocyte-rich fraction의 탐식성을 측정한 결과, 어느 경우에도 증강효과는 나타나지 않았다. 이상의 결과로부터 CLA 중 CLA mixture, 10t-12c CLA, 9c-11t CLA가 돼지 말초혈액 탐식세포에 대하여 탐식증강효과를 가지고 있으며 이러한 증강효과는 직접적이라기보다는 CLA에 의해 활성화된 단핵구세포에서 분비되는 탐식촉진인자를 함유한 가용성 물질에 의해 autocrine 또는 paracrine 양상으로 탐식세포에 작용하는 것으로 생각되었다.

주요어 : 돼지, conjugated linoleic acid, 말초혈액탐식세포, 탐식능.