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Immunoenhancing Effects of Conjugated Linoleic Acid on Chemotactic Activity of Porcine Peripheral Blood Polymorphonuclear Cells

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Abstract : Immunoenhancing effects of conjugated linoleic acid (CLA) isomers (10t-12c CLA, 9c-11t CLA, CLA mixture, 9c-11c CLA and 9t-11t CLA) on chemotactic activity of porcine peripheral blood polymorphonuclear cells (PMN) were examined. The chemotactic activity of PMN was evaluated by a modified Boyden chamber assay. CLA isomers at higher concentration of 50 to 200 μM exhibited a low viability of cells by trypan blue exclusion. CLA isomers were used at concentration of 20 μM showing no cytotoxic effect and high cell viability. CLA isomers themselves were not active or slight chemotactic for PMN. But culture supernatant from mononuclear cells (MNC) treated with 10t-12c CLA, 9c-11t CLA and CLA mixture except for 9c-11c CLA and 9t-11t CLA enhanced remarkably chemotactic activity of porcine PMN. PMN migration by culture supernatant from MNC treated with CLA mixture was found to be true chemotaxis by checkboard assay. This migration was also induced by porcine recombinant interleukin (rIL)-8. PMN chemotaxis caused both culture supernatant from MNC treated with CLA mixture and porcine rIL-8 was inhibited in a dose-dependent manner by addition of anti-porcine IL-8 polyclonal antibody. Therefore, these results strongly suggested that CLA (10t-12c CLA, 9c-11t CLA and CLA mixture) could stimulate porcine MNC to release an IL-8 like chemotactic activity.

Key words **:** chemotactic activity, conjugated linoleic acid (CLA), peripheral blood, mononuclear cells (MNC), polymorphonuclear cells (PMN), porcine

Introduction

Conjugated octadecadienoic acids were 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[10]. The predominant natural isomer in animals is the 9c-11t isomer[4,5,22]. CLA can be also synthesized in the laboratory from pure linoleic acid or from sources high in linoleic acid such as sunflower oil, safflower oil or corn oil by a reaction using heat and basic conditions. With this method, 95% linoleic acid (9c-12c) substrate can be converted into 9c-11t (~43%) and 10t-12c octadecadienoic acid (~44%)[5].

Anticarcinogenic activity was triggered by the accidental discovery[18] in later isolated CLA isomers as being responsible for the anticarcinogenic action. Since then, the inhibitory action of CLA was demonstrated in carcinogenesis models including epidermal[2,9], mammary[12,13,30] and gastrointestinal carcinomas[12,17]. CLA in physiological concentration inhibited proliferation of several human cancer cell lines *in vitro*[25,27,28]. The 9c-11t CLA and 10t-12c CLA in numerous isomers were known to possess biological activity[6]. The 10t-12c CLA increased blastogenesis of lymphocyte[8]. 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[7]. The 9c-11t CLA and 10t-12c CLA isomers may be equally effective in inhibiting carcinogenesis[13].

There have been a few studies on the effects of CLA on immune function. When CLA was added to porcine lymphocyte culture *in vitro*, it increased cytotoxic activity of lymphocyte and killing ability of murine macrophage[17]. Rats fed a diet containing 0.5% CLA had enhanced macrophage phagocytosis and foot pad swelling in response to phytohemagglutinin (PHA)[7]. Mice fed 0.3% and 0.9% CLA had increased *in vitro* lymphocyte proliferation in response to PHA but not concanavalin A or lipopolysaccharide (LPS). Interleukin (IL)-2 production in mice was also stimulated by CLA[32]. In spite of these observations, the immunomodulatory effects of CLA on nonspecific immune responses have not been well investigated yet. Thus, in the current study the immunoenhancing effect of CLA isomers on chemotactic activity of porcine PMN was examined.

Materials and Methods

Animals

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 hrs light/dark cycle. Pigs were fed on commercial diet and tap water.

Reagents

CLA mixture (Nu-Chek-Prep Corporation, MN, USA), 10t-12c CLA, 9c-11t CLA, 9c-11c CLA and 9t-11t CLA (Matreya, Inc, PA, USA) were commercially purchased. Porcine recom-

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binant (pr) IL-8, goat anti-pr IL-1β polyclonal antibodies (pAb) (IgG) and goat anti-pr IL-8 pAb (IgG) (R&D systems Inc, Minneapolis, MN, USA) were also commercially purchased.

MNC 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; Pharmacia Inc, Piscataway, NJ, USA). After centrifugation at 400 × g for 40 minutes at room temperature, the MNC in interface between PBS plus plasma and Ficoll-hypaque solution was harvested. 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 MNC layer. Cell viability determined by trypan blue dye exclusion methods, always exceeded 98%. All cells were resuspended in RPMI 1640 (Gibco Co, Grand Isiand, NY, USA) supplemented with 2 mM L-glutamine, 0.02 mg/ml of gentamicin and 5% fetal bovine serum (Gibco Co, Grand Isiand, NY, USA) and finally adjusted to 2×10^6 cells/ml.

Culture supernatants

The MNC at a density of 2×10^6 cells/ml in a well of a 24-well tissue plate or TC dishes (21.5 cm^2; Nunc Co, Naperville, IL, USA) was incubated with a concentration of 20 μM/ml of CLA isomers (CLA mixture, 10t-12c CLA, 9c-11t CLA, 9c-11c CLA and 9t-11t CLA) for 24 hrs at 37°C under 5% CO_2-humidified atmosphere. All supernatants were collected by centrifugation (5,000 g for 30 minutes), filtered with 0.45 μm-pore size membrane filter and stored at - 70°C until use for assay.

Chemotaxis assay

A modified Boyden chamber assay was used to measure chemotaxis. A nitrocellulose filters with 120-μm thick and 3.0-μm pore size (Nihon Millipore, Yonezawa, Ibaraki, Japan) was placed on the top of well of the lower chamber that previously filled with 200 μl of CLA isomers at various concentrations, culture supernatants from MNC treated with CLA isomers, and pr IL-8, respectively. The controls were medium alone or culture supernatants from untreated cells in the lower compartment. 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. The membrane filters were immediately taken out, stained with hematoxylin, and mounted on glass slide. The migrated distance of PMN through the filter toward the other side was measured by microscopy. The chemotactic responsiveness of input PMN was evaluated as absolute distance (μm/45 minutes ± SE) in the directional migration of PMN in response to chemoattractants.

Checkerboard assay

Checkerboard assay was carried out according to the method of Zigmond and Hirsch[38].

Neutralization test

Anti-pr IL 8 pAb and control IgG diluted with various concentrations were mixed with the culture supernatant from MNC treated with CLA mixture. The mixed culture supernatants were placed for 30 minutes at room temperature and the chemotactic activity for PMN was also evaluated.

Data analysis

The Student's t test was used for statistical significance determinations. All data expressed mean ± SE.

Results

Cell viability of MNC and PMN

The trypan blue exclusions were executed to examine the viability of MNC and PMN treated with CLA isomers at concentrations ranging from 10 to 200 μM for 24 hrs. MNC at concentration of more than 50 μM of CLA mixture, 10t-12c CLA, and 9c-11t CLA showed low viability or was extirpated (Table 1). PMN at concentration of more than 100 μM above CLA isomers was also extirpated (Table 2). Thus, CLA isomers were used at concentration of 20 μM showing high cell viability and no cytotoxic effect.

Direct effect of CLA isomers on PMN chemotaxis

As shown in Fig 1, the direct treatments of all CLA isomers at concentrations ranging from 0 to 100 μM have no enhancing effects on chemotaxis of PMN when compared with control.

Table 1. Cell viability (%) of MNC treated with CLA isomers for 24 hrs

CLA isomers	Concentration (μM)					
	0	10	30	50	100	200
CLA mixture	96±0.57	98±0.88	94±0.57	75±1.20	-	-
10t-12c CLA	95±0.57	98±0.66	94±0.57	76±1.45	-	-
9c-11t CLA	97±0.33	97±0.88	96±0.88	77±2.08	-	-
9c-11c CLA	99±0.57	98±0.33	98±0.57	95±0.57	-	-
9t-11t CLA	99±0.33	97±0.57	95±0.88	91±2.18	94±0.88	93±0.33

Table 2. Cell viability (%) of PMN treated with CLA isomers for 24 hrs

CLA isomers	Concentration (μM)					
	0	10	30	50	100	200
CLA mixture	96±1.15	98±0.33	97±0.57	96±0.57	-	-
10t-12c CLA	95±0.57	98±0.57	98±0.33	94±1.00	-	-
9c-11t CLA	97±0.33	97±0.57	96±0.57	91±1.15	-	-
9c-11c CLA	99±0.57	98±0.33	98±0.33	98±0.57	84±2.15	-
9t-11t CLA	99±0.57	97±0.33	95±0.57	91±1.15	94±0.33	98±0.57

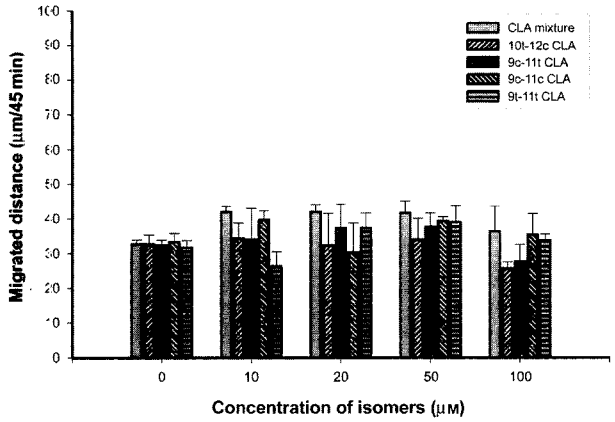

Fig 1. The direct effects of CLA isomers on porcine PMN chemotaxis. The values represent mean ± SE (n=3).

Effect of culture supernatants from MNC treated with CLA isomers on PMN chemotaxis

The culture supernatant from MNC treated with CLA mixture enhanced remarkably PMN chemotaxis as shown in Fig 2. This enhancement showed a peak at 1/16 dilution (6.25%) of culture supernatant. Also, culture supernatant from MNC treated with 10t-12c CLA and 9c-11t CLA showed a significant enhancement ($P < 0.01$) of PMN chemotaxis as compared to that of control. These enhancements were dose-dependent manners. However, culture supernatant from MNC treated with 9c-11c CLA and 9t-11t CLA showed no enhancing effect on chemotaxis of PMN.

Checkerboard assay

In order to determine whether PMN migration by culture supernatants from MNC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA is random movement (chemokinesis) or directed cell movement (chemotaxis), the checkerboard assay using the culture supernatant from MNC treated with CLA mixture was performed. As shown in Table 3, PMN migration strongly depended on both the increase of a concentration gradient of culture supernatant in lower chamber and the decrease of a concentration gradient of culture supernatant in upper chamber. This indicated that chemotactic activity of PMN by culture supernatant treated with CLA isomers was true chemotaxis but not random migration.

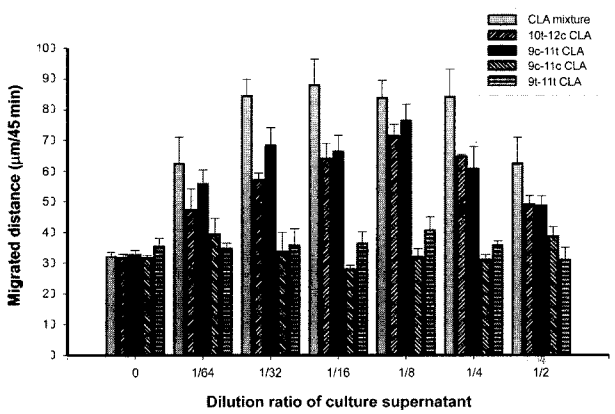

Fig 2. Chemotactic response of PMN to culture supernatant from MNC treated with CLA isomers (20 μM). The values represent mean ± SE (n=3). *P<0.05, **<0.01, compared to control (0)

Table 3. Checkerboard assay of porcine PMN migration to culture supernatant from MNC treated with CLA mixture

Dilution of culture supernatant on upper chamber	Dilution of culture supernatant on lower chamber			
	0	1/64	1/16	1/4
0	37.5±1.16	63.6±6.98	77.1±4.72	83.0±1.79
1/64	29.5±1.25	43.8±5.87	56.1±5.21	69.6±3.08
1/16	29.3±0.54	36.0±3.58	41.5±8.64	58.6±3.48
1/4	21.5±4.29	28.6±4.50	31.0±3.69	33.1±3.70

Culture supernatant from MNC treated with CLA mixture (20 μM) for 24 hrs and diluted with RPMI 1640 medium.
Values of migrated distance (μm/45minutes) represent as mean ± SE of three determinations.

Chemotactic response of PMN by pr IL-8

The chemotactic activity of PMN to pr IL-8 was examined. As shown in Fig 3, pr IL-8 enhanced significantly the chemotactic activity of PMN at concentration of 1 to 100 nM ($P < 0.01$). This activity of PMN to pr IL-8 at concentration of 10 nM was equivalent to that of culture supernatant (6.25%) from MNC treated with CLA mixture.

Neutralization test with anti-pr IL-8 pAb

To examine whether the enhanced chemotactic activity of PMN to culture supernatant from MNC treated with CLA mixture is due to IL-8, the neutralization test using the anti-pr IL-8 pAb was performed. The chemotactic activities of PMN to both culture supernatant from MNC treated with CLA mixture and pr IL-8 were inhibited ($p < 0.05$ to 0.01) by addition of anti-pr IL-8 pAb at concentration of 0.1 to 5.0 μg/ml when compared with those of positive controls (6.25% of

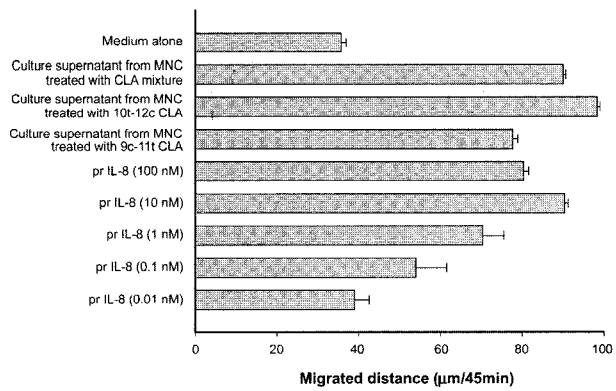

Fig 3. Chemotactic response of PMN to pr IL-8 at concentration of 0.01 to 100 nM and culture supernatant (6.25%) from MNC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA. The data represent mean ± SE (n = 3). *P<0.05, **<0.01, compared to control (medium alone)

culture supernatant from treated with CLA mixture and 10 nM of pr IL-8). However, in the examination of the possibility of nonspecific inhibition for immunoglobulin isotype, IgG, of anti-pr IL-8 pAb, any chemotactic activity of PMN to culture supernatant from MNC treated with CLA mixture and pr IL-8 was not inhibited by addition of high concentration of 5.0 μg/ml of control IgG (Fig 4).

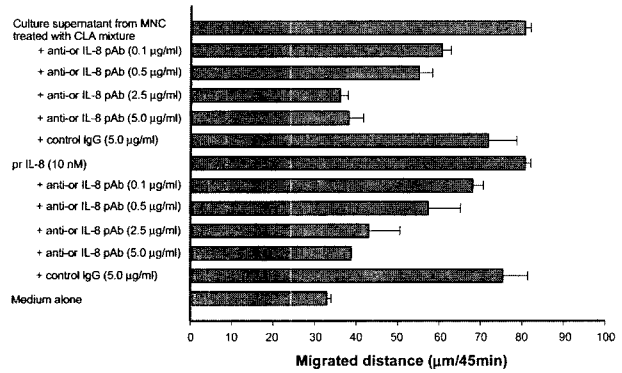

Fig 4. Effect of anti-pr IL-8 pAb on porcine PMN to culture supernatant (6.25%) from MNC treated with CLA mixture and pr IL-8 (10 nM). The values represent mean ± SE (n = 3). *P<0.05, **<0.01, compared to culture supernatant (6.25%) from MNC treated with CLA mixture and pr IL-8 (10 nM).

Discussion

In the previous study associated with adipocyte, the concentration of CLA isomers used was 50 μM[22]. However, in the present study MNC and PMN at concentration of 50 μM to 200 μM of CLA isomers (CLA mixture, 10t-12c CLA and 9c-11t CLA) were extirpated and showed the reduced viabilities. Therefore, CLA isomers were used at concentration of 20 μM showing high cell viability and no cytotoxic effect. It was assumed that CLA isomers at higher concentration show cytotoxicity in peripheral blood leukocytes unlike adipocytes since leukocytes showing a rapid life cycle are sensitive to active CLA isomers.

CLA is not a single compound but a mixture of geometrical and positional isomers with *cis* and *trans* conjugated double bonds located at the 9,11 carbons and 10,12 carbons[9]. These CLA isomers are synthesized by base isomerization of linoleic acid. It was of interest that the presence and absence of chemotactic activity for PMN in culture supernatant from MNC treated with CLA isomers differs in classes of CLA isomers. Culture supernatants from MNC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA enhanced remarkably chemotactic activity for PMN. Whereas culture supernatants from MNC treated with 9c-11c CLA and 9t-11t CLA failed to induce chemotactic response of PMN. Although it is difficult to explain the reason for presence and absence of chemotactic activity, it was thought that biological activity of CLA may be also differs in the kinds of its isomers and that CLA isomers show the multiple effect according to biochemical mechanisms. In fact, the 9c-11t CLA and 10t-12c CLA were known to possess biological activity[20]. CLA mixture was also predominantly consisted of 9c-11t octadecadienoate (43%) and 10t-12c octadecadienoate (44%)[19].

The present results showed that CLA isomers themselves were not effective on the migration of freshly prepared porcine PMN. Leukotriene B_4 (LTB_4)[35] and platelet activating factor (PAF)[1] are potent chemoattractants and activators of leukocytes and involved in inflammatory diseases. It has been also suggested that CLA isomers can be incorporated into membrane phospholipids and may replace arachidonic acid[11,12]. CLA isomers inhibit LTB_4 and PAF in arachidonic acid metabolites[29,31] and reduce the release of prostaglandin (PG) E_2 from antigen-challenged lung, trachea, and bladder in the guinea pig[11,12]. Therefore, it was presumed that CLA isomers have no change *in vitro* metabolite production of arachidonic acid in porcine PMN and have directly no effects on chemotaxis of PMN.

Activated monocytes and lymphocytes release many factors associated with host defense and inflammation and directly induce chemotactic response for phagocytes[14]. The representative chemokines produced activated MNC are known as IL-8 and IL-1. However, although the culture supernatant of LPS-stimulated monocytes enhanced a neutrophil chemotactic activity, highly purified or recombinant IL-1 did not affect chemotactic activity of neutrophils[24]. The findings that culture supernatants from MNC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA were able to enhance the chemotactic activity for PMN might suggest that enhancing effect of CLA on chemotactic response for porcine PMN is mediated by soluble product(s) released by CLA-stimulated MNC. Chemotactic activity for porcine PMN by pr IL-8 was also equivalent to that of culture supernatant from MNC treated with CLA mixture. In addition, pAb against pr IL-8 inhibited PMN chemotaxis which was enhanced by both culture supernatants of MNC treated with CLA mixture and pr IL-8. It was reported that the chemotactic activity for PMN in culture supernatant from MNC exposed to egg white derivatives (EWD), one of immunostimulators, is identified as IL-8 with molecular weight of 6 to 8 kDa[15,33,34]. Therefore, it could be thought that soluble products, which is produced by MNC in response to CLA isomers, will be associated with the IL-8-like chemotactic factor(s) of PMN.

IL-8 is a potent chemokine for PMN which stimulates its chemotaxis[36]. The cellular responses mediated by IL-8 are transmitted via specific receptors[26]. High affinity receptors for IL-8 (67 kDa and 59 kDa) were identified on the surface of human neutrophils that bind IL-8 but not IL-1 and tumor necrosis factor (TNF)-α[3,23]. When PMN and monocytes were activated in the initial stage of immune response, the small amount of secreted IL-8 stimulates effectively the release of such as IL-1, IL-6 and TNF-α from mononuclear cells and promotes phagocytic activity[37].

The elucidation of porcine IL-8-like chemotactic factor(s)

produced from porcine MNC treated with CLA will be considerably important in the study of animal cytokines as well as immunostimulators. This study suggests that CLA could enhance nonspecific immunity in porcine PMN by releasing IL-8-like chemotactic factor from MNC.

Conclusion

The current study was undertaken to examine the immunostimulating effect of CLA isomers (CLA mixture, 10t-12c CLA, 9c-11t CLA, 9c-11c CLA and 9t-11t CLA) on porcine PMN chemotaxis. CLA isomers at higher concentration of 50 to 200 μM exhibited a low viability of cells by trypan blue exclusion. Thus, CLA isomers were used at concentration of 20 μM showing high cell viability and no cytotoxic effect. CLA isomers themselves were not chemotactic for PMN. However, culture supernatants from MNC treated with CLA mixture, 10t-12c CLA and 9c-11t CLA but not 9c-11c CLA and 9t-11t CLA enhanced porcine PMN chemotaxis. The migration of PMN to culture supernatant from MNC treated with CLA mixture was authentic chemotaxis by checkerboard assay Chemotactic activity of PMN was also induced by pr IL-8. This enhanced chemotactic activity of PMN to both culture supernatant from MNC treated with CLA mixture and pr IL-8 is inhibited in a dose-dependent manner by addition of anti-pr IL-8 pAb. These results strongly suggest that CLA isomers (CLA mixture, 10t-12c CLA and 9c-11t CLA) have an enhancing effect on chemotaxis of PMN, which may be mediated through IL-8-like factor produced by CLA-stimulated MNC.

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돼지 말초혈액 다형핵 백혈구의 유주성에 있어서 conjugated linoleic acid의 면역증강효과

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요 약 : 돼지 말초혈액 다형핵 백혈구(polymorphonuclear cell; PMN)의 유주성에 있어서 conjugated linoleic acid (CLA) 이성체(CLA mixture, 10t-12c CLA, 9c-11t CLA, 9c-11c CLA 및 9t-11t CLA)의 면역증강 효과를 검토하였다. PMN에 대한 유주성은 Boyden chamber 변법으로 측정하였다. CLA 이성체들을 고농도(50~200 μM)로 사용하였을 경우 말초혈액 단핵구 세포(mononuclear cell; MNC) 및 PMN의 cell viability가 감소되거나 세포가 사멸하였다. 따라서 cell viability가 높고 세포독성을 나타내지 않는 20 μM 농도로 유주활성 실험을 하였다. CLA 이성체들은 돼지 말초혈액 PMN의 유주활성에 직접적인 효과는 없었다. CLA 이성체로 배양한 MNC의 배양상층액 중 CLA mixture, 10t-12c CLA 및 9c-11t CLA 처리군에서는 PMN의 유주활성이 현저하게 증강되었으나 9c-11c CLA 및 9t-11t CLA로 배양한 MNC 배양상층액에서는 PMN의 유주활성이 나타나지 않았다. 이러한 유주성 증강효과는 checkerboard assay를 실시한 결과 진성의 유주활성이었다. 유주성 인자인 porcine recombinant (pr) interleukin (IL)-8을 이용하여 돼지 PMN에 대한 유주성을 검토한 결과, pr IL-8에 의한 PMN의 유주활성은 CLA로 배양한 MNC 배양상층액에 의한 것과 동등한 활성을 보였다. 또한 CLA로 배양한 MNC 배양상층액의 PMN에 대한 유주성을 anti-pr IL-8 pAb를 사용하여 중화반응을 실시한 결과, CLA mixture로 배양한 MNC 배양상층액에 의해 증가된 PMN의 유주활성은 anti-pr IL-8 pAb 첨가에 의해 억제되어, 본 유주활성은 MNC에서 분비되는 IL-8 으로 인한 것임을 강하게 시사하였다. 이상의 결과로부터 CLA 중 CLA mixture, 10t-12c CLA 및 9c-11t CLA 이성체가 돼지 말초혈액 다형핵 백혈구의 유주활성에 증강효과를 가지고 있으며, 이러한 증강효과는 CLA로 자극된 MNC에 의해 생성되는 IL-8 樣 인자에 의한 것임을 알 수 있었다.

주요어 : 말초혈액 단핵구 세포, 다형핵 백혈구, 유주활성, 돼지, conjugated linoleic acid