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Modulatory Effects of Ionized Alkali Mineral Complex (IAMC) on mRNA Expression of Porcine Cytokines

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ABSTRACT. It has been recognized that ionized alkali mineral complex (IAMC)-fed farm animals demonstrate higher weight gains but less incidence of diseases than the unfed ones. However, how these beneficial effects in the IAMC-fed animals are induced has not yet been elucidated clearly. In this study, porcine peripheral blood mononuclear cells (PBMC) were cultured for 4, 24, and 48 hr in the presence of IAMC, and the effects of IAMC on mRNA expression of porcine cytokines were evaluated via a reverse transcription-polymerase chain reaction (RT-PCR). Expression levels of IL-4, IL-6, and IL-10 in IAMC-treated cells were usually higher than those in the untreated ones. However, IAMC-treated cells demonstrated a reduced expression of IL-2. In addition, expression of IFN-γ was generally reduced in the cells treated with IAMC. The expression of IL-12 p35 and IL-12 p40 was not detectable in both the untreated and the IAMC-treated cells. Therefore, these results indicate that IAMC has immunomodulatory effects *in vitro* on the expression of porcine Th1-and Th2-type cytokines.

KEY WORDS: immunomodulatory effect, ionized alkali mineral complex, mRNA expression, porcine cytokine.

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Cytokines, classified into two types in mouse and human, act as important immune modulator regulating cellular and humoral immune responses [11, 12]. While cytokines produced by Th1 cells, such as IFN-γ, IL-2, and IL-12, promote a cell-mediated immune reaction, those secreted by Th2 cells, such as IL-4, IL-5, IL-6, and IL-10, are involved in the induction of a humoral immune response [12]. Th1- and Th2-type cytokines have mutual inhibitory relations for regulating the immune homeostasis [12]. For example, IFN-γ inhibits the production of Th2-type cytokines, however, IL-10 suppresses the expression of Th1-type cytokines including IFN-γ and IL-12 [3, 5, 9, 12].

Ionized alkali mineral complex (IAMC) has been used to improve natural immunity against the pathogenic organisms and weight gains of animals in the field farms particularly in Korea. IAMC-treatment to pigs induces increases in $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, MHC class II-expressing cells, and IgM^+ B lymphocytes [14]. Supplementation of IAMC to the pigs vaccinated with hog cholera virus (HCV) vaccine demonstrated an increase in HCV-specific antibody production [13]. Although it is generally accepted that IAMC-fed animals exhibit an improved health state, how the IAMC-treatment exerts the beneficial effect in animals has not yet been defined. Therefore, our objective was to examine the cytokine production profile in IAMC-treated cells in hopes of elucidating the mechanism of the improved immunity induced by IAMC. In this study, we adapted RT-PCR used in other cytokine expression studies [4, 15] to examine the modulatory effect of IAMC on the expression of porcine cytokines, including IFN-γ, IL-2, IL-4, IL-6, IL-10, and IL-12 [1, 3, 6, 7, 10, 18].

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MATERIALS AND METHODS

Experimental animals and cell preparation: Approximately 10 m*l* of blood was collected from two of 3–4 months old pigs using a sodium heparin-treated vacutainer (Becton Dikinson, Franklin Lakes, NJ) to prevent coagulation. Peripheral blood mononuclear cells (PBMC) were separated from whole blood through centrifugation at 900 × g for 30 min in the presence of Histopaque 1077 (Sigma, St. Louis, MO) placed at the bottom of the conical tube. The isolated PBMC were washed twice with RPMI 1640 media (Gibco BRL, Grand Island, NY) via centrifugation at 300 × g for 10 min and were cultured in 75-cm^2 tissue culture flasks (Nunc, Denmark).

Treatment of PBMC with PHA and ionized alkali mineral complex: PBMC were stimulated with phytohemagglutinin M form (PHA M) (2% v/v) for 4 hr at 37°C-humidified incubator in the presence of 5% CO_2. Total RNA from PHA M-treated cell was extracted for cDNA synthesis. The synthesized cDNA was used as a template in PCR to demonstrate the specificity of PCR reaction by amplifying cytokine genes. Powerfeel^{TM}, the brand name of IAMC, was kindly provided by NEL, Biotech. Inc. (Ansung, Korea). PBMC were divided into four groups. The first group cells were used as untreated control cells that were cultured in the complete RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 50 μg/m*l* gentamicin, 5 × 10^{-5} M 2-mercaptoethanol, and 2 mM L-glutamine. The second, third, and fourth group cells were cultured in the complete RPMI media containing 1:300-, 1:600-, and 1:1200-diluted IAMC, respectively. All cells were cultured in a 37°C-humidified incubator for 4, 24, and 48 hr, and

Table 1. Primer sets used to amplify cytokine genes

Primer sets	Primer sequences	Sizes of PCR Products	GenBank Accession
IFN-γ	5'-ATGAGTTATACAACTTATTTCTTAG-3' 5'-TTATTTTGATGCTCTCTGGCC-3'	501 bp	S63967
IL-2	5'-ATGTATAAGATGCAGCTCTTG-3' 5'-TCAAGTCAGTGTTGAGTAGATG-3'	465 bp	X56750
IL-4	5'-ATGGGTCTCACCTCCCAACTG-3' 5'-TCAACACTTTGAGTATTTCTCCTTC-3'	402 bp	X68330
IL-6	5'-ATGAACTCCCTCTCCACAAGC-3' 5'-CTACATTATCCGAATGGCCCTC-3'	639 bp	M80258
IL-10	5'-ATGCCCAGCTCAGCACTGC-3' 5'-TCAGTTCTTCCTCATCTTCATC-3'	528 bp	L20001
IL-12 p35	5'-ATGTGTCCGCTGCGCAAC-3' 5'-TTAGGAAGAATTCAGATAGCTC-3'	669 bp	L35765
IL-12 p40	5'-ATGCACCTTCAGCAGCTGGTTG-3' 5'-CTAATTGCAGGACACAGATGC-3'	975 bp	U08317
Cyclophilin A	5'-ATGGTTAACCCCACCGTCTTC-3' 5'-GTTTGCCATCCAACCACTCAG-3'	376 bp	F14571

their mRNAs were extracted for analysis.

Isolation of total RNA and synthesis of cDNA: Total RNAs were isolated from the control and IAMC-treated cells using Trizol reagent (Gibco BRL, Grand Island, NY). After isolation, the RNAs of two pigs were combined and treated with 2 units of RNase-free DNase (Promega, Madison, WI) for 30 min at 37°C to remove the residual DNA, and the RNAs were purified again with Trizol reagent. Single-stranded cDNA was made using Superscript Preamplification System for First Strand cDNA Synthesis Kit (Gibco BRL, Grand Island, NY). Briefly, 5 μg of RNA was incubated for 50 min at 42°C in the presence of 10 $\times$ PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP, 0.1 M DTT, and 200 units of Superscript II RT, and the samples were treated with 2 units of RNase H for 20 min at 37°C. The synthesized single-stranded cDNA was used in PCR as a template.

PCR amplification: Expression of each cytokine gene was examined via PCR amplification (45 cycles) with its respective cytokine-specific primer set (Table 1) and a template cDNA under the following conditions: IFN-γ; 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s: IL-2; 94°C for 30 s, 53°C for 30 s, and 72°C for 45 s: IL-4; 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s: IL-6; 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s: IL-10; 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s: IL-12 p35; 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s: IL-12 p40; 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s. Cyclophilin A[19] was used as a housekeeping gene control, and its constant expression was demonstrated through the PCR amplification under the following conditions: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

Determination of cytokine gene expression: All PCR products were analyzed by electrophoresis in 1.0% agarose gel containing ethidium bromide dye under UV light. Densities of the PCR products were determined with a Gel Documentation System (Bio-Rad, Hercules, CA). Cytokine-specific band density was divided by cyclophilin A-specific band density in order to normalize the expression levels of cytokine genes.

RESULTS

Specificity of PCR reactions and expression of cyclophilin A: Under each gene-specific condition, PCR amplification with cDNA made from mRNA of PHA M-stimulated PBMC produced cytokine-specific DNA bands, including IFN-γ, IL-2, IL-4, IL-6, IL-10, IL12 p35, and IL-12 p40, and also a cyclophilin A-specific band (Fig. 1). This result indicated that the PCR conditions used in this study were suitable for detection of expressed cytokine mRNA in porcine PBMC. As a house-keeping gene, expression levels of cyclophilin A were consistent in the control and the IAMC-treated cells (data not shown).

Expression of IFN-γ, IL-2, and IL-12: Expression levels of IFN-γ generally seemed to be variable under different conditions. The expression of IFN-γ in cells treated with IAMC for 4 and 24 hr was lower than that in the control cells. However, IFN-γ expression of the cells treated with 1:600-diluted IAMC for 48 hr was higher than that of the control cells (Fig. 2A). Expression levels of IL-2 in the IAMC-treated cells were always lower than those of the control cells (Fig. 2B). These results indicated that IAMC-treatment of PBMC seemed to induce a reduction of IL-2 expression. Neither IL-12 p35 nor IL-12 p40 expression was demonstrated in both the control and IAMC-treated cells (data not shown).

Expression of IL-4, IL-6, and IL-10: Neither the untreated control nor IAMC-treated cells cultured for 4 and 24 hr exhibited expression of IL-4 (Fig. 2C). However, an elevated expression of IL-4 could be seen in the cells treated with IAMC for 48 hr (Fig. 2C). The expression of IL-4 in the control cells was lower than that of the IAMC-treated cells. Expression levels of IL-6 were always higher in cells treated with IAMC for 4, 24, and 48 hr than those in the untreated control cells cultured for the same periods (Fig.

Fig. 1. Specificity of PCR. Genes of cytokine and cyclophilin A were amplified by PCR with cDNA synthesized from mRNA of PHA-stimulated PBMC for 4 hr. M, DNA marker; Lane 1, IFN-γ (501 bp); Lane 2, IL-2 (465 bp); Lane 3, IL-4 (402 bp); Lane 4, IL-6 (639 bp); Lane 5, IL-10 (528 bp); Lane 6, IL-12 p35 (669 bp); Lane 7, IL-12 p40 (975 bp); Lane 8, cyclophilin A (376 bp).

2D). Expression of IL-10 was higher in the untreated control cells than that in the IAMC-treated cells incubated for 4 hr (Fig. 2E). However, higher expression of IL-10 appeared in the cells treated with IAMC for 24 and 48 hr than in the untreated control cells (Fig. 2E).

DISCUSSION

In this study, cytokine expression patterns were examined in the porcine PBMC cultured in the presence of ionized alkali mineral complex (IAMC). IAMC-treatment demonstrated an immunomodulatory effect on the expression of cytokines. Cytokines play as a potent modulator of immune and inflammatory responses, and each cytokine conducts its function through synergistic or antagonistic interactions with other cytokines [2]. Porcine lymphocytes isolated from IAMC-fed pigs exhibited higher expression of $CD4^+$, $CD8^+$, and MHC class II molecules, and higher proliferation rates than those in untreated control group cells [20].

While IAMC-treated porcine PBMC demonstrated increased expression levels of IL-4, IL-6, and IL-10, those of IFN-γ and IL-2 were reduced in the same cells. In mice, IL-4 and IL-10 caused inhibition of Th1-type cytokine expression, including IFN-γ, IL-2, and IL-12 [5, 8, 9]. Por-

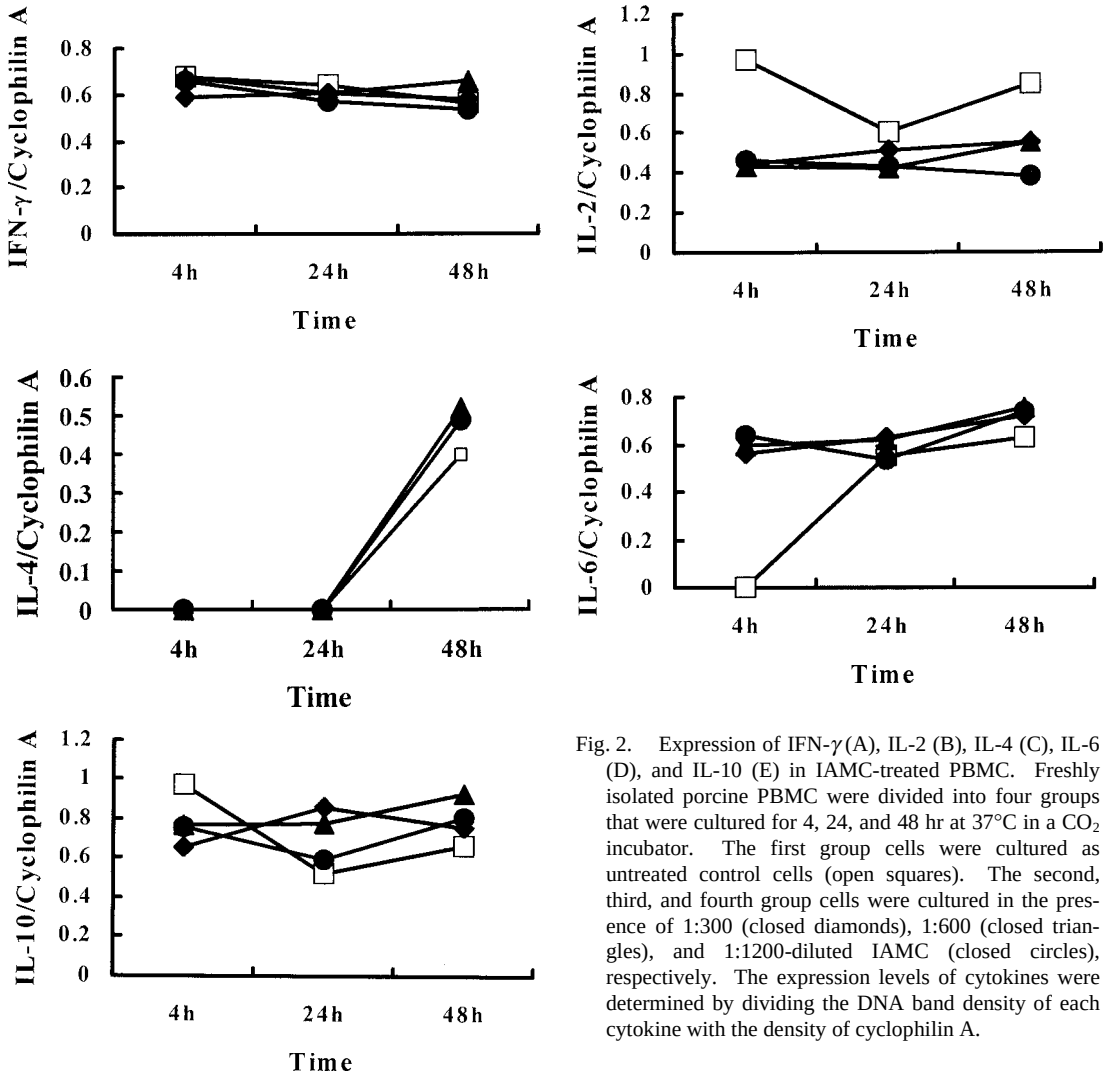

Fig. 2. Expression of IFN-γ (A), IL-2 (B), IL-4 (C), IL-6 (D), and IL-10 (E) in IAMC-treated PBMC. Freshly isolated porcine PBMC were divided into four groups that were cultured for 4, 24, and 48 hr at 37°C in a CO_2 incubator. The first group cells were cultured as untreated control cells (open squares). The second, third, and fourth group cells were cultured in the presence of 1:300 (closed diamonds), 1:600 (closed triangles), and 1:1200-diluted IAMC (closed circles), respectively. The expression levels of cytokines were determined by dividing the DNA band density of each cytokine with the density of cyclophilin A.

cine IL-10 also induced a suppression of IFN-γ production [3]. However, it was shown that IL-4 induced enhanced expression of IL-10 mRNA [17]. In this study, it was evident that a little increased expression of IL-4 appeared 48 hr after IAMC-treatment. Expression of IL-10 in the IAMC-treated cells also increased after 24 and 48 hr incubation. IL-6 expression levels in the IAMC-treated cells were always higher than those in the untreated control cells. These results imply that IAMC-treatment of porcine PBMC may induce enhanced expression of Th2-type cytokines. In addition, it may be speculated that expression of Th1-type cytokines is decreased through an IL-10-mediated antagonistic mechanism.

Th2-type cytokines are involved in the induction of a humoral immune response *in vivo*, stimulating the antibody production [12, 16]. It was demonstrated that IAMC-treated pigs immunized with hog cholera live vaccine produced a higher HCV-specific antibody than the control pigs [13]. Therefore, taken together, it could be concluded that IAMC-treatment induces the humoral immune response both *in vivo* and *in vitro*.

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