

Coenzyme A contained in mothers' milk is associated with the potential to induce atopic dermatitis

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Abstract

T_H2 adjuvant activity can be qualitatively and quantitatively evaluated using a mixed lymphocyte reaction and by changes in the intracellular cyclic adenosine 3',5'-monophosphate concentration, using human dendritic cells *in vitro*. The current study shows that mothers, whose children (*n* = 55) developed atopic dermatitis (AD) within 6 months after birth, often demonstrate a higher T_H2 adjuvant activity in their milk, in comparison to those whose children did not develop such symptoms. Such an activity was recovered in a liquid phase of mothers' milk and was eluted as a single fraction by reversed-phase HPLC. Further analysis of this fraction by mass spectrometry showed that signals originating from a factor with a molecular weight of 767.53 are observed, exclusively in milk with a high T_H2 adjuvant activity. The mass is exactly that of Coenzyme A (CoA), and indeed, a low concentration of CoA exhibited T_H2 adjuvant activity both *in vitro* and *in vivo*. Moreover, mesenteric lymph node non-T cells obtained from mice that were orally treated with CoA led allogeneic naive CD4⁺ T cells to differentiate into T_H2. Furthermore, the oral administration of CoA induced rough skin, hyperplasia of the epidermis, hypergranulosis in the spinous layer and the thickening of the stratum in mice. These data collectively indicate that some of the patients with AD were exposed to mothers' milk carrying high T_H2 adjuvant activity right after birth, which may be attributable to presence of CoA contained in the milk.

Keywords: atopic dermatitis, coenzyme A, dendritic cell, T_H2 adjuvant

Introduction

Dendritic cells (DCs) are antigen-presenting cells specialized to activate naive T lymphocytes and initiate primary immune responses. The different classes of specific immune responses are driven by the biased development of pathogen-specific effector T-cell subsets—that is T_H1, T_H2 and T_H17 cells, that activate different components of cellular and humoral immunity. T_H cell differentiation is critical for achieving proper immune responses, and imbalances in either the function or the activity of these cell types are responsible for many immune diseases, including autoimmunity, cancer and allergy (1, 2).

DCs reside in an immature state in many nonlymphoid tissues such as the skin, the intestine, or airway mucosa, which are under high exposure of pathogens and chemicals. DCs, which take up pathogens, develop their maturation processes, migrate to the T-cell areas of secondary lymphoid

organs and interact with naive T cells. TCR stimulation and co-stimulation allow naive T_H cells to develop into protective effector cells, normally accompanied by the high-level expression of selective sets of cytokines. The balance of these cytokines and the resulting class of immune response strongly depend on the conditions under which DCs are primed for the expression of the T-cell-polarizing molecules (3).

The ligands for many isoforms of toll-like receptors, including certain nucleic acids, LPS and fungus-derived glycoprotein molecules, alter the DC function and induce T_H1 differentiation in an antigen nonspecific manner (4). In this process, IL-12 produced by DCs is clearly correlated with sensitization of T_H1 lymphocytes *in vitro* and *in vivo* among the factors that have been shown to influence the T_H1–T_H2 balance (5).

On the other hand, DCs matured in the presence of prostaglandin E_2 (PGE_2), histamine or forskolin induce the differentiation of naive $CD4^+$ T cells toward T_H2 via the cyclic adenosine 3',5'-monophosphate (cAMP) cascade (6-9). *In vitro* assay systems have been established to evaluate T_H1/T_H2 adjuvant activities, using mixed lymphocyte reaction (MLR) (10) and intracellular cAMP concentration of antigen-presenting cells (11). The present study found that mothers, whose children developed atopic dermatitis (AD) within 6 months after birth, carry higher T_H2 adjuvant activity in their milk, in comparison to those whose children did not.

Methods

Cohorts

A prospective birth cohort of 900 newborn infants was established in Chiba University Hospital and JFE Kawatetsu-Chiba Hospital, from January 2007 to May 2008. All participants received a questionnaire after their infants were born. Data on parental allergic disease and various exposures were obtained. The parents answered questionnaires with the main focus on symptoms related to eczema lasting for at least 2 months when the infant was 6 months age. The complete response rate on the 6-month questionnaire was 68%, from which 55 AD(+) and 55 AD(−) subjects were randomly selected. The frozen stock of these mothers' milk was subjected to T_H2 adjuvant activity assay. All participants provided written informed consent to participate in the study. This study was approved by the ethics committee of Chiba University Graduate School of Medicine.

Sampling and storage of mothers' milk

The mothers' milk specimens were obtained 4 days after birth and then were immediately frozen (-80°C) for long-term storage to avoid LPS contamination. The milk samples were thawed immediately before testing and were centrifuged ($10\,000 \times g$, 10 min) to obtain surface lipid, interface liquid and cell-containing pellets. Sampling 20% of preparations exhibited trace LPS levels.

Measurement of intracellular cAMP

THP-1 cells were cultured in RPMI 1640 media, supplemented with 10% FCS, 1% penicillin and streptomycin and 1% L-glutamine in a humidified incubator under 5% CO_2 at 37°C . THP-1 cells were exposed to 50 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA) for 48 h, washed and then incubated at 37°C in the presence of 1 mM 3-isobutyl-1-methyl-xanthine. Ten minutes later, the cells were stimulated with 30% mothers' milk (AD: $n = 55$; non-AD: $n = 55$) for 10 min and then were lysed with cell lysis buffer (Molecular Devices). In some experiments, the THP-1 cells treated with 50 ng ml^{-1} PMA for 48 h were exposed to $10\text{ }\mu\text{M}$ PGE_2 . The intracellular cAMP levels were determined using the Catch-Point(tm) cAMP fluorescent assay kit (Molecular Devices), according to the manufacturer's instructions. In some experiments, delta (Δ)-cAMP was calculated by subtracting the cAMP concentration in the media obtained without an adjuvant.

Preparation of human Mo-DCs and T lymphocytes

Human Mo-DCs (monocyte-DCs) and $CD45RA^+$ naive $CD4^+$ T cells (>99% purity) were prepared as previously described (12). The study using peripheral blood specimens obtained from healthy volunteers was approved by the Saitama Medical University Ethics Committee.

DC-mediated T-cell differentiation assay

Immature Mo-DCs were stimulated with 0-10% of the liquid fraction from mothers' milk or $0-10\text{ }\mu\text{g ml}^{-1}$ Coenzyme A (CoA). The cells were incubated with either the liquid fraction or the CoA for 2 days, and then the cellular components were further co-cultured with HLA-DR-nonshared allogeneic naive $CD4^+$ T cells to induce an MLR in RPMI 1640 medium supplemented with 10% human serum for 6-8 days. Thereafter, the T cells were re-stimulated with anti-CD3 and anti-CD28 antibodies (BD Pharmingen, USA). Culture supernatants were harvested after 24 h to be assayed for $\text{IFN}\gamma$ and IL-5 by an ELISA using $\text{IFN}\gamma$ and IL-5 ELISA kits (R&D Systems, USA). This study was approved by the ethics committee of Saitama Medical University.

HPLC analysis

The liquid phase of mothers' milk was loaded onto a $4.5 \times 250\text{ mm}$ C8 reversed-phase (RP)-HPLC column (Shiseido, Japan) equilibrated with 0.06% TFA. The column was eluted at a flow rate of 1.0 ml min^{-1} at room temperature in a gradient of increasing acetonitrile (ACN) concentration in 0.052% TFA with the A214 and A280 continuously monitored. The fractions were concentrated and dried using the Speed Vac (Savant).

Mass spectrometry

Mass spectra were recorded on an LCMS-IT-TOF mass spectrometer (Schimadzu Corporation, Kyoto, Japan) equipped with an electrospray ionization source. The sample was pulse injected at a flow rate of 0.2 ml min^{-1} with buffer containing 50% ACN and 0.1% formic acid mixture in water. The electrospray voltage was set to 4.5 kV and capillary temperature was 200°C . The nebulizer was set at 1.5 l min^{-1} nitrogen. The spectra were acquired by scanning over a 200-1000 mass/charge range every 0.3 s for 2 min. The LCMS Solution software package was used for the integration of every spectrum for the scanning time and also for calculating the mass in the expected range.

Mice

SJL/J, BALB/c, Nc/Nga and HR-1 mice were purchased from Charles River, Japan, SLC Inc. and Hoshino Experimental Animal Center, respectively. All mice were bred in a pathogen-free environment in the Division of Laboratory of Animal Medicine. All studies using mice were approved by the Ethics Review Committee for Animal Experimentation of Saitama Medical University.

Murine MLR analysis followed by oral administration of CoA

SJL/J mice (H-2^s) were administrated with 400 micrograms per day per mouse of CoA orally. $CD3\epsilon^-$ cells were obtained

from the mesenteric lymph node cells after 2 days using CD3 ϵ^- cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD62L $^+$ naive CD4 $^+$ T cells were obtained from the spleen of an Nc/Nga mice (non-H-2 ^{s}) by CD4 $^+$ T-cell isolation kit and CD62L $^+$ T-cell isolation kit (Miltenyi Biotec) on the same day. Thereafter, 1×10^4 of CD3 ϵ^- cells and 1×10^5 of allogeneic CD62L $^+$ naive CD4 $^+$ T cells were co-cultured in a 96-well flat-bottomed culture plate to induce an MLR in RPMI 1640 medium supplemented with 10% FCS for 7 days. Next the T cells were re-stimulated with anti-CD3 and anti-CD28 antibodies (Biolegend, USA). Culture supernatants were harvested after 16 h to be assayed for IFN γ , IL-4 and IL-5 by an ELISA using IFN γ , IL-4 and IL-5 ELISA kits (R&D Systems).

Ovalbumin peptide-specific immune responses in the presence of CoA

BALB/c mice were intra-peritoneally administrated with or without 2 mg of CoA in the presence of 2 μ g of ovalbumin peptide (OVA) (Sigma-Aldrich) on days 0 and 10 and were orally administered with CoA or PBS, three times a week. On day 20, mice were sacrificed to obtain spleen cells and sera. Mononuclear cells were isolated by Ficoll-Paque (GE healthcare) centrifugation. Then the 6×10^6 per well of mononuclear cells were cultured in a 12-well flat-bottomed culture plate with 1 μ g ml $^{-1}$ OVA in DMEM medium supplemented with 10% FCS for 7 days. Then culture supernatants were harvested to be assayed for IFN γ , IL-4 and IL-13 by an ELISA using IFN γ , IL-4 and IL-13 ELISA kits (R&D Systems).

To determine OVA-specific antibody concentration in serum samples, ELISA plates were coated overnight with 10 mg ml $^{-1}$ OVA in PBS. After washing with PBS, serum samples were incubated for 2 h, followed by probing with HRP-conjugated anti-mouse IgG2a and IgG1, respectively (Bethyl Laboratories Inc.). After washing with PBS, OVA-specific IgG2a and IgG1 were detected by TMB substrate solution (Bethyl Laboratories Inc.). Murine serum obtained after immunization with 2 μ g of OVA + 2 mg of aluminum hydroxide was pooled and used as a positive control. Anti-OVA IgG2a and IgG1 concentration in this sample were defined as 1000 local units per milliliter.

Histological analysis in mice followed by oral administration of CoA

HR-1 mice were fed with a special diet and either used as control or were administrated 400 micrograms per day per mouse of CoA orally, starting from 4 weeks of age. After 11 and 16 weeks of treatment, the mice were sacrificed, and skin samples were obtained, fixed in 10% neutral buffered formaldehyde, embedded in paraffin and stained with hematoxylin and eosin.

Statistical analysis

Comparisons between the sets of two groups were performed using Student's two-tailed *t*-test, while sets of more than two groups were compared by analysis of variance. Some experiments were statistically analyzed using the Mann-Whitney's *U*-test.

Results

Association of T_H2 adjuvant activity of mothers' milk with AD

This study first evaluated the concentration of cAMP within PMA-stimulated THP-1 cells in response to mothers' milk. As shown in Fig. 1, some AD-associated milk samples demonstrated an increased cAMP formation in THP-1 cells ($P = 0.0108$); however, none of the mothers' milk fed to healthy infants demonstrated such an activity. These results raised the possibility that the milk of some mothers of infants with AD therefore may demonstrate T_H2 adjuvant activities.

T_H2 adjuvant activity is in a liquid phase of the milk

The mothers' milk was separated into three fractions by centrifugation, i.e. a cell-containing pellet, interface liquid and surface lipids. These fractions were co-incubated with PMA-treated THP-1 cells to evaluate cAMP-elevating activity. cAMP-elevating activity was efficiently recovered in the liquid fraction (Fig. 2A). The activity remained intact even after filtration for sterilization. The pellet and the lipid fractions as well as the mock incubation and AD(−) mother's milk exhibited no activity. The T_H1/T_H2 cytokine profiles were examined in a DC-mediated T-cell differentiation assay using human monocyte-derived DCs (Mo-DCs), via stimulation with the filtered liquid phase of the milk. Milk preparations were co-incubated with DCs alone; T cells were not exposed to the milk. LPS and forskolin were used as positive controls for a T_H1 and a T_H2 adjuvant, respectively (data not shown). The liquid phase of the milk led to high concentration of IL-5 and a high IL-5:IFN γ ratio, as compared with a negative control (Fig. 2B). These results suggest that the milk with cAMP-elevating activity in PMA-derived THP-1 cells indeed possesses a T_H2-inducing activity by acting on DCs. The experiment was repeated twice and the same results were obtained.

Semi-purification and mass spectrometry of the T_H2 adjuvant activity

The liquid phase of the milk was semi-purified by using C8 RP-HPLC. The cAMP-inducing activity of 30-s fractions was

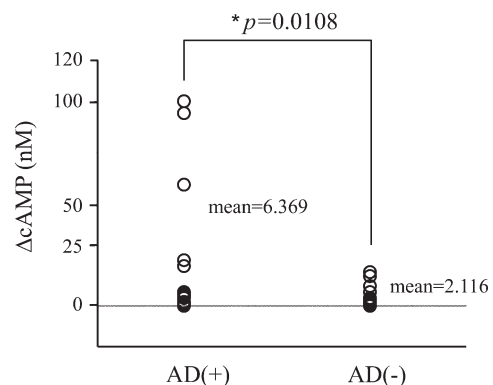


Fig. 1. cAMP-elevating and T_H2-polarizing activity of mothers' milk. THP-1 cells were treated with 50 ng ml $^{-1}$ PMA. Forty-eight hours later, the cells were washed and then exposed to 30% mothers' milk. The intracellular cAMP levels were determined using the CatchPoint(tm) cAMP fluorescent assay kit, as described in Methods. Delta (Δ)-cAMP was calculated by subtracting the cAMP concentration in the media obtained without an adjuvant.

determined, and the 11.0- to 11.5-min fraction of high cAMP-inducing milk exclusively exhibited the highest activity, whereas that of low activity did not (Fig. 3). The experiment

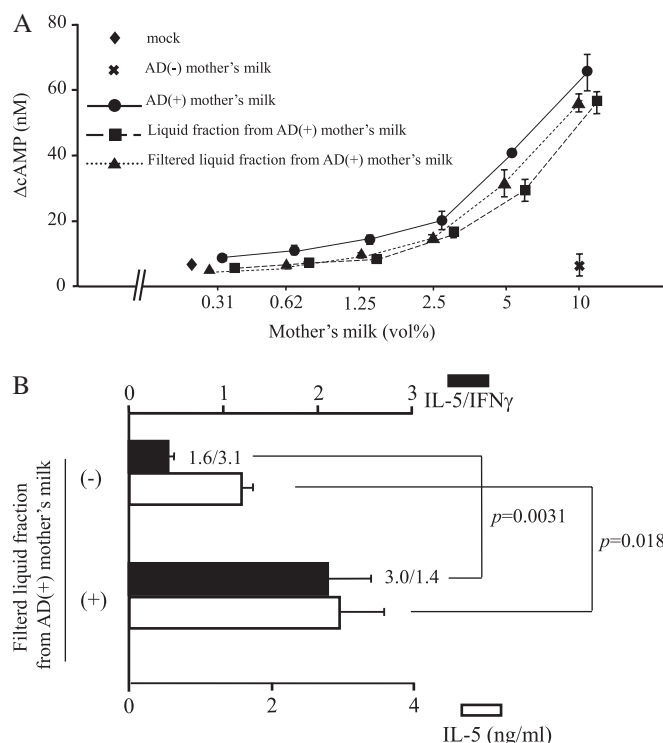


Fig. 2. T_H2 adjuvant activity is in a liquid phase of the milk. (A) Milk samples were separated into surface lipid, interface liquid and pellet fractions by centrifugation and subjected to cAMP analysis. Mothers' milk: solid line; liquid fraction: bold dotted line; filtered liquid fraction: thin dotted line. (B) Immature DCs were incubated with (final 10%) or without filtered liquid fraction of AD(+) mother's milk and were subjected to MLR analysis. Numbers in the figure indicate the absolute values (nanograms per milliliter) of cytokines.

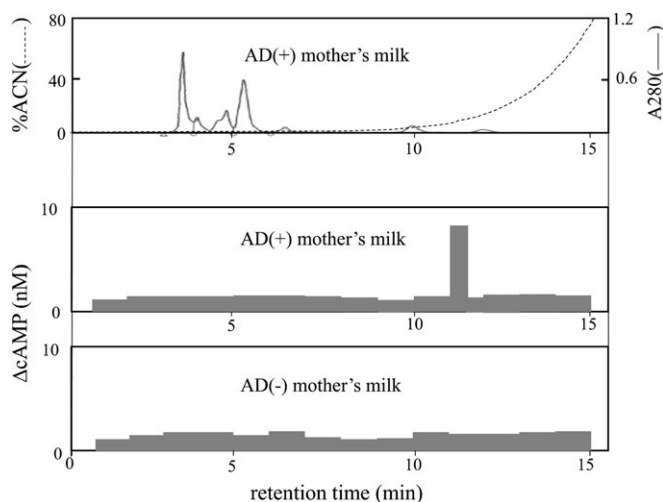


Fig. 3. Purification of cAMP-elevating activity by RP-HPLC. The liquid phase of mothers' milk was loaded on C8 RP-HPLC. Solid line, A280; dotted line, % ACN. Thirty-second fractions were concentrated and dried with the Speed Vac and then were subjected to the cAMP analysis.

was repeated with three different donors for each group, and the reproducible results were obtained. This fraction contained little A280 signal, thus indicating that there is little protein. The 15- to 25-min fractions exhibited significant A280 signals but no cAMP-inducing signals (data not shown). This 11.0- to 11.5-min fraction was analyzed by mass spectrometry. Among six major signals detected, the $M_i = 384.7719$ and $M_i + 1 = 385.2754$ ($M_i + 1 - M_i = 0.5035$) signals were detected with high-activity milk but not with low-activity milk, corresponding to a molecular mass of 767.5292 (Table 1), which is the mass of CoA. An examination of two other milk preparations exhibited similar results, thus suggesting a possibility that CoA in the milk contributes to the T_H2 adjuvant activity.

T_H2 adjuvant activity carried by CoA

The T_H2 adjuvant activity of CoA was tested by the changes in intracellular cAMP concentration and by an MLR using human DCs *in vitro*. PMA-treated THP-1 cells exhibited elevated intracellular cAMP when co-incubated with 10–30 $\mu\text{g ml}^{-1}$ (13–39 μM) of CoA (Fig. 4A). Furthermore, Mo-DCs that were pre-treated with 1 $\mu\text{g ml}^{-1}$ CoA induced allogeneic naive CD4^+ T cells to differentiate into T_H2 (Fig. 4B). The experiments were repeated three times and the same results were obtained.

Oral administration of CoA induces differentiation into T_H2 using MLR in mice

Further experiments examined whether CoA exhibits T_H2 adjuvant activity *in vivo*. SJL/J and Nc/Nga were treated orally with 400 micrograms per day per mouse of CoA. This dose roughly corresponds to the oral intake of CoA by an infant whose mother's milk exhibits the highest T_H2 adjuvant activity shown in Fig. 1 if infant's body weight (3 kg), milk intake (800–1200 ml day^{-1}) and the highest CoA concentration of mother's milk (30 $\mu\text{g ml}^{-1}$) are considered. CD3e^- cells were isolated from the mesenteric lymph node 2 days after CoA administration and co-cultured with allogeneic CD62L^+ naive CD4^+ T cells. This MLR study significantly led to high concentration of IL-4 and IL-5, as compared with a control group (water without CoA; Fig. 5A). Although no significant statistical difference was observed in IFNγ (Fig. 5A), IL-4:IFNγ and IL-5:IFNγ ratios were markedly higher in the treated mice (Fig. 5B). The experiment was repeated twice and the same results were obtained.

CoA induces differentiation of T_H2 -type immune responses *in vivo*

Further experiments were performed to test whether CoA induces differentiation into T_H2 in OVA-specific immune responses. BALB/c mice were intra-peritoneally administered with or without 2 mg of CoA in the presence of 2 μg of OVA. Intra-peritoneal administration of CoA + OVA significantly led to high concentration of IL-4 and IL-13 and low concentration of IFNγ, as compared with a control group, which was immunized with OVA alone (Fig. 5C). We next tested whether OVA-specific antibody production skewed toward T_H2 -type responses. In this experiment, effects of oral administration of CoA were also evaluated. As shown in Fig. 5(D), although no significant statistical difference was

Table 1. Mass spectrometry analysis of mothers' milk

Mother's milk of									
AD(+) infant					AD(-) infant				
Mi	Mi + 1	Mi + 1 - Mi	V	MW	Mi	Mi + 1	Mi + 1 - Mi	V	MW
216.1228	217.1273	1.0045	1	215.1155	216.1231	217.1149	0.9918	1	215.1158
599.3896	600.3924	1.0028	1	598.3823	599.3895	600.3933	1.0038	1	598.3822
316.2127	317.2143	1.0016	1	315.2054	316.2127	317.2147	1.0020	1	315.2054
384.7719	385.2754	0.5035	2	767.5292			Trace		
430.2440	431.2476	1.0036	1	429.2367	430.2454	431.2577	1.0123	1	429.2381
485.3589	486.3613	1.0024	1	484.3516	485.3585	486.3604	1.0019	1	484.3512

V, valence number; MW, molecular weight.

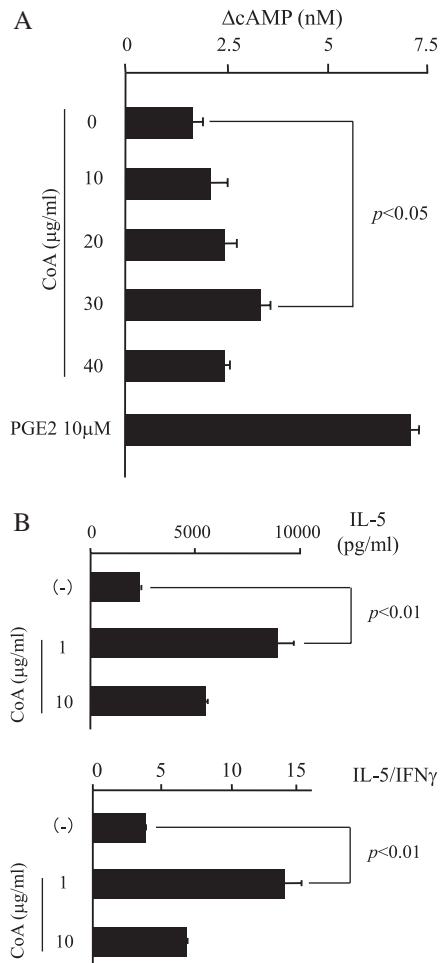


Fig. 4. cAMP-elevating and Th₂-polarizing activity of CoA. (A) THP-1 cells were treated with 50 ng ml⁻¹ PMA. Forty-eight hours later, cells were washed and then exposed to 0–40 μg ml⁻¹ of CoA or 10 μM PGE₂ as a control. Intracellular cAMP levels were determined using the CatchPoint(tm) cAMP fluorescent assay kit, as described in the Methods. (B) The CD14⁺ cells were stimulated with IL-4 and granulocyte macrophage colony-stimulating factor for 5 days and the cells were harvested as immature Mo-DCs. After washing, immature Mo-DCs were incubated with 0–10 μg ml⁻¹ of CoA. Two days after the incubation with CoA, cellular components were further co-cultured for 7 days with HLA-DR-nonshared allogeneic CD4⁺ naive T cells to induce an MLR. Thereafter, the T cells were re-stimulated with anti-CD3 and anti-CD28 antibodies. The culture supernatants were harvested after 24 h to be assayed for IFNγ and IL-5 by an ELISA.

observed in OVA-specific IgG2a, oral or intra-peritoneal administration of CoA in the presence of OVA significantly led to high concentration of IgG1, as compared with a control group (OVA alone). Interestingly, combination of oral and intra-peritoneal administration with CoA in the presence of OVA led to markedly high concentration of IgG1, suggesting that both oral and intra-peritoneal CoA exhibit Th₂ adjuvant activity and each has an additive effect.

Oral administration of CoA macroscopically induces rough skin and microscopically induced hyperplasia of the epidermis, hypergranulosis in the spinous layer and the thickening of the stratum in HR-1 mice.

Further experiments were performed to test whether CoA induces AD-like symptoms *in vivo*. The HR-1 mice were treated orally with 400 micrograms per day per mouse of CoA. At 15 weeks of age, no visible differences were observed in the CoA-treated mice and untreated mice. However, the histological examination of CoA-treated mice indicated slight hyperplasia of the epidermis compared with untreated mice (Fig. 6A). As shown in Fig. 6(B), at 20 weeks of age, the skin of CoA-treated mice was rougher than that of untreated mice. The CoA-treated mice also showed more prominent hyperplasia of the epidermis compared with untreated mice (Fig. 6C). Interestingly, hypergranulosis in the spinous layer and thickening of the stratum were also apparent in these mice compared with untreated mice.

Discussion

The skin of a patient with AD is abnormally and easily affected by irritants, food and environmental allergens. It also becomes vulnerable to surface infections caused by bacteria. The association of food allergy with AD has now been clearly demonstrated (13, 14). Many common food allergens such as milk, eggs, nuts, cheese, tomatoes, wheat, yeast, soy and corn can trigger an allergic reaction. Shreffler *et al.* (15) demonstrated that molecules in some food allergens drove DCs to enhance the Th₂ polarization. They contribute to the development of an allergy, not only as allergens that react with specific IgE but also as adjuvants that react with DCs. Such adjuvant activities have also been reported with pollens (16) and parasites (17, 18). The magnitude of Th₂ adjuvant activity is associated with increased levels of cAMP in PMA-derived THP-1 cells (11). The short incubation period (10 min) for this assay allows for the testing of non-sterile materials such as live bacteria (19) or non-sterile milk, as reported in the current study.

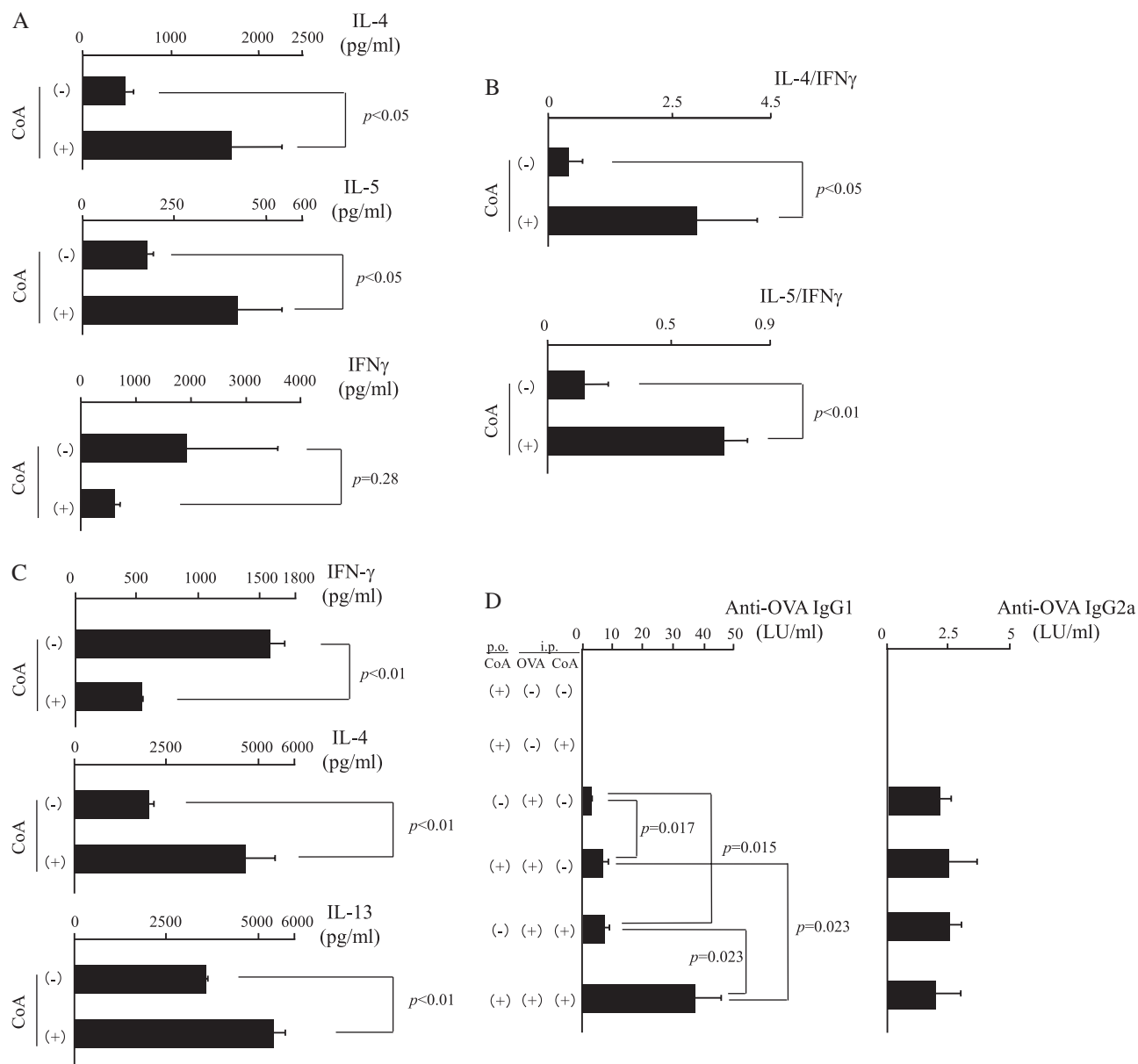


Fig. 5. Th2-polarizing activity of CoA *in vivo*. (A) SJL/J mice (H-2^s) were orally administrated with or without 10–20 $\mu\text{g kg}^{-1} \text{ day}^{-1}$ of CoA in water. After two days, CD3 ϵ^{-} cells were obtained from mesenteric lymph node cells in SJL/J mice and naive CD4 $^{+}$ T cells were obtained from the spleen of an Nc/Nga mouse (non-H-2^s), as described in the Methods. Then 1×10^4 of CD3 ϵ^{-} cells and 1×10^5 of allogeneic CD62L $^{+}$ naive CD4 $^{+}$ T cells were co-cultured in a 96-well flat-bottomed culture plate to induce an MLR in RPMI 1640 medium supplemented with 10% FCS for 7 days. Thereafter, the T cells were re-stimulated with anti-CD3 and anti-CD28 antibodies. The culture supernatants were harvested after 16 h to be assayed for IFN- γ , IL-4 and IL-5 by an ELISA using IFN- γ , IL-4 and IL-5 ELISA kits. (B) IL-4:IFN- γ and IL-5:IFN- γ ratios. (C) BALB/c mice were intra-peritoneally administrated with or without 2 mg of CoA in the presence of 2 μg (of) OVA. Mononuclear cells from spleen cells were cultured in a 12-well flat-bottomed culture plate with 1 $\mu\text{g ml}^{-1}$ OVA. The culture supernatants were harvested after 7 days to be assayed for IFN- γ , IL-4 and IL-13 by an ELISA. (D) BALB/c mice were intra-peritoneally and/or orally administrated with or without CoA in the presence or absence of OVA. Anti-OVA IgG1 and IgG2a concentrations in serum samples were determined by an ELISA, as described in the Methods.

The current study showed that some mothers, whose children developed AD within 6 months after birth, carry high Th2 adjuvant activity in their milk obtained 4 days right after birth. Such activity was not observed in mothers' milk fed to healthy infants. A mass spectrometry analysis using AD (+) infants' milk exhibited signals that originated from factors with a molecular weight of 767.53, exclusively in milk with a high Th2 adjuvant activity. The mass corresponds to that of CoA, which

indeed increased cAMP level in PMA-derived THP-1 cells and induced Th2 polarization in an MLR system and OVA-specific immune response. Although an increased cAMP was most efficiently induced by 30 $\mu\text{g ml}^{-1}$ CoA, as low as 1 $\mu\text{g ml}^{-1}$ of CoA was sufficient in an MLR study for the optimal Th2 polarization. This discrepancy may be attributable to the fact that (i) PMA-stimulated THP-1 cells are not physiological DCs and (ii) PMA-stimulated THP-1 cells and monocyte-derived

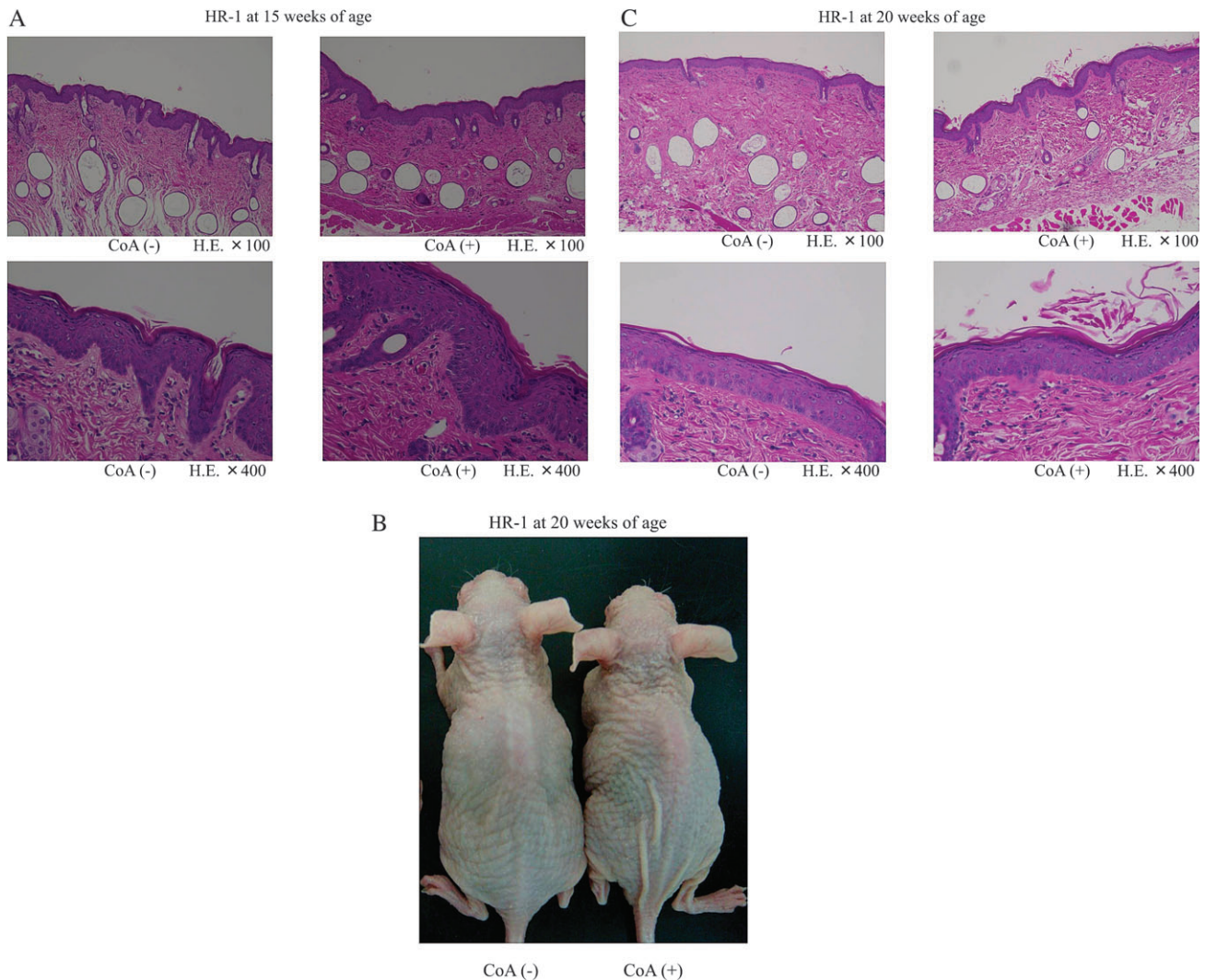


Fig. 6. CoA induces hyperplasia of epidermis *in vivo*. HR-1 mice were fed with a special diet and either used as control or were administrated 400 micrograms per day per mouse of CoA orally, starting from 4 weeks of age. After 11 and 16 weeks of treatment, the mice were sacrificed and skin was obtained and histological examination was performed as described in the Methods. (A) At 15 weeks of age, the skin sample obtained from non-treated mice (left) and CoA-treated mice (right) and stained with hematoxylin and eosin (HE). (B) Visible observation of non-treated mice (left) and CoA-treated mice (right) at 20 weeks of age. (C) At 20 weeks of age, the skin sample obtained from non-treated mice (left) and CoA-treated mice (right) and stained with HE.

immature DCs are incubated with adjuvant preparations, for 10 min and 48 h, respectively, thereby generating a discrepancy in an integrated biological effect.

Sears *et al.* (20) reported that more children who were breastfed were atopic at all ages from 13 to 21 years to cats ($P = 0.0001$), house dust mites ($P = 0.0010$) and grass pollen ($P < 0.0001$) than those who were not. More children who were breastfed reported current asthma at each assessment between age 9 ($P = 0.0008$) and 26 years ($P = 0.0008$) than those who were not. The results from observational birth cohort studies, case-control studies and one cluster-randomized intervention trial have generally failed to demonstrate a protective effect of breastfeeding on outcomes of AD, allergic sensitization, wheezing or asthma (21). Although these studies reported that AD correlates with breastfeeding, it is unclear whether mothers' milk itself

contributes to AD development (22). The current results suggest a direct effect of mothers' milk with the development of AD. It is also possible to speculate that the mothers' milk contains not only of CoA but also of other components with T_H2 adjuvant activity. However, it is highly likely that CoA is a major component in mothers' milk carrying T_H2 adjuvant activity, because (i) CoA activity in milk samples ($18.8 \mu\text{g ml}^{-1}$) from five AD(+)'s, which induce maximum cAMP concentration, was higher ($P = 0.03$) than that in milk samples ($6.0 \mu\text{g ml}^{-1}$) from AD(-)'s, which induced minimum cAMP concentration, and (ii) inhibition of CoA activity by oxidation of mothers' milk markedly decreased T_H2 adjuvant activity (data not shown).

CoA is composed of units derived from cysteine, pantothenic acid and ATP. It plays important roles in the synthesis and oxidation of fatty acids, pyruvate oxidation in the citric

acid cycle and many other biological processes. One of the main functions of CoA is the carrying and transfer of acyl groups. One of the most important acyl groups transferred is the acetate group, in which case the molecule is called acetyl-CoA. The acetyl group eventually finds itself incorporated into a variety of molecules such as cholesterol, acetylcholine, melatonin, heme and the TCA cycle intermediates. The molecular mechanism by which CoA increases the intracellular cAMP is still not fully understood. However, it is well known that cAMP increases the transcription of CoA synthase, thereby generating a positive feedback loop (23).

The current study showed that CoA demonstrated Th2 adjuvant activity in mice by oral administration. Interestingly, both oral and intra-peritoneal administration with CoA is able to induce high Th2 adjuvant activity in OVA-specific immune system. The intestinal mucosa has a unique and complicated immune system composed of a variety of cell populations. Atarashi *et al.* (24) reported that ATP derived from commensal bacteria activates a unique subset of lamina propria cells, CD70^{high}CD11c^{low} cells, thus leading to the differentiation of Th17 cells. The administration of ATP exacerbates a T-cell-mediated colitis model with enhanced Th17 differentiation. It has been reported that CoA modulates the differentiation and stratification of keratinocytes and also modulates sebocyte differentiation (25). Our data suggest that the CoA-treated mice had modulated epidermal differentiation. The ceramide/cholesterol ratio in the stratum corneum is known to be low in the stratum of patients with AD (26). It is speculated that cholesterol metabolism is important in the stratification of the epidermis (27). The activity of HMG-CoA reductase and acetyl-CoA carboxylase are key for cholesterol synthesis (28). Our data show that administration of CoA contributes to the epidermal differentiation and keratinization of the epidermis in an *in vivo* model. To more convincingly show that CoA is responsible for the observed changes, antibodies to CoA should be used to inhibit or remove activities from milk samples. Unfortunately, specific antibodies to CoA are not available at present. Furthermore, we are now trying to establish a method for artificial feeding starting immediately after birth for mice, because the oral administration of CoA to lactating mice did not increase the CoA concentration in the milk (data not shown). To initiate a study on CoA in foods, we have determined CoA concentration in dry milk products. The concentrations showed wide variation, ranging from <0.5 to 13.4 µg ml⁻¹. Further studies are needed to determine whether CoA contained in dry milk products is associated with the development of AD.

In summary, the current study demonstrated that CoA in mothers' milk was associated with the risk for developing infant AD. Studies are now underway to determine: (i) why the CoA concentration in milk varies markedly among individual and (ii) whether or not appropriate intervention can result in a preventive effect on the development of AD.

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