Studies on IgA Induction in Intestine and Mammary Glands of Mammals

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Contents

Abbreviations

ASC; Antibody-secreting cells

CCL25; Chemokine ligand 25 (thymus-expressed chemokine)

CCL28; Chemokine ligand 28 (mucosa-associated epithelial chemokine)

GAPDH; glyceraldehydes-3-phosphate dehydrogenase

HPLC; High-performance liquid chromatography

Ig; Immunoglobulin

MAdCAM-1; Mucosal addressin cell adhesion molecule-1

ROS; Reactive oxygen species

RA; All-*trans* retinoic acid

RAR; Retinoic acid receptor

RXR; Retinoid X receptor

VCAM-1; Vascular cell-adhesion molecule-1

dpp; days postpartum

pIgR; Polymeric immunoglobulin receptor

Chapter I

General Introduction

Mortality and morbidity of neonates continue to be major problems in calves, and their most common disease is diarrhea, which can cause growth retardation and death of calves. Successful neonatal health depends on many factors related to management and nutrition, but the improvement of immune system is required for preventing diarrhea. Passive immunity is critical to the survival and health of neonates, and colostrum is a source of nutrients and immune components for neonatal calves (Blum 2006). In mammalian species, the adaptive immune system of the mother can provide passive protection to the suckling newborn through immunoglobulin (Ig) ingested in the mother's milk (Stoliar *et al.* 1976). The transfer of maternal Ig to the neonate provides transient immune protection to pathogens previously encountered by the mother, and contributes to the dramatically reduced mortality levels in children who are breast fed compared with those who are formula fed in developing countries (Feachem & Koblinsky 1984).

The basic structure of Ig comprises four polypeptide chains (two light chains and two heavy chains), which are connected by disulfide bridges. The light chains contain one variable region and one constant region, whereas the heavy chains have three or four constant regions. Structural differences in heavy chains are the basis of five classes of Ig (IgA, IgD, IgE, IgG and IgM) in humans and rodents (Mix *et al*. 2006). IgG is the most abundant Ig isotype in bovine colostrum and can be transported across the intestinal epithelial cells into the neonatal circulation at 24 h after birth (Weaver *et al*. 2000). IgA is the most abundant Ig isotype in mucosal secretions and provides

protection against microbial antigens at mucosal surfaces in guts, but colostral IgA is very low (Stelwagen *et al*. 2009; Ishikawa *et al*. 1992). There is a direct relationship between the amounts of colostrum and serum IgG in calves, and mortality rate of calves with serum IgG concentrations less than 10 mg/mL is more than twice as high as that of calves with higher IgG concentrations in Holstein cows (Hopkins & Quigley 1997). The improvement of colostral IgG and IgA in Japanese Black cows is needed for the appropriate calf health and immune system, because the lower transfer of IgG and IgA from colostrum to neonatal calves was found in Japanese Black calves at 2 days of age (Yasumatsuya *et al*. 2013).

Immunoglobulins are produced by plasma cells, which are derived from the precursor B cells in the bone marrow (Driessen & van der Burg 2011). Through a complex differentiation process, the precursor B cells differentiate into the immature B cells and start to express IgM molecules on the cell surface (Fuentes-Pananá *et al*. 2004). The immature B cells can also express IgD molecules due to the alternative splicing of heavy-chain gene transcripts (Kerr *et al*. 1991). Secreting IgM can be found at normal levels in serum in germ-free mice, whereas the serum IgG and IgA levels are about 5% of normal mice, suggesting that the secretion of IgG and IgA requires the stimulation of antigens (Bos *et al*. 1989; Schliephake & Schimpl 1996). IgM^+ B cells migrate into germinal centers in lymphoid tissues, interact with T cells and antigen-presenting cells (such as macrophages and dendritic cells) and initiate a class switch recombination (Liu *et al*. 1991). Class switch recombination can replace the genes that encode constant regions in Ig heavy chains, and consequently switch the isotype of B cells from IgM to IgA, IgG or IgE (Stavnezer-Nordgren & Sirlin 1986). Class switch recombination is regulated by a complex signaling pathway and various

cytokines are involved in. Cross-linking of CD40 on the surface of the B cell and CD40L on the surface of the T cell activates the T cell to release cytokines and induces the class switching together with specific cytokines (Quezada *et al*. 2004). Cytokines such as IL4, IL10, IL-21 and IFN-γ are involved in the class switching to IgG (Gascan *et al*. 1991; Kühn *et al*. 1991; Pène *et al*. 2004; Malisan *et al*. 1996), whereas TGF-β and IL10 can induce IgA (Brière *et al*. 1994; Lebman *et al*. 1990). Additionally, antigen presenting cells also express the B lymphocyte stimulators such as BAFF and APRIL ligands, and can activate a CD40-like pathway to initiate a T cell-independent class switching to IgA and IgG (Litinskiy *et al*. 2002).

Gut-associated lymphoid tissue is the largest immunological tissue in the body. Peyer's patches are the main site for the generation of IgA^+ B cells, and plasmablasts differentiating from IgA^+ B cells preferentially home in on the gut lamina propria through the thoracic duct and blood by the expression of homing ligands and receptors (Fagarasan & Honjo 2003; Sigmundsdottir & Butcher 2008; Ertesvåg *et al*. 2009). Chemokines are transmembrane proteins that play important roles in innate and acquired immunity (Morteau *et al*. 2008; Mora *et al*. 2006). Chemokine ligand CCL25 (thymus-expressed chemokine) is selectively expressed in the small intestine and its receptor CCR9 is expressed on the surface of almost all T cells in the small intestine and a fraction of IgA antibody-secreting cells (ASC) (Hieshima *et al*. 2004). The interplay of CCL25 and CCR9 is likely to play a critical role in the recruitment of developing thymocytes (Wurbel *et al*. 2000), and the selective expression of CCL25 in the small intestine underlies the homing of $CCR9^+$ intestinal memory T cells to the small intestine (Papadakis *et al*. 2000).

IgA plasma cells in the mammary glands in mice are derived from the lymphoid

cells in gut-associated lymphoid tissue by homing to the mammary glands (Mora *et al*. 2006). Homing of ASC to the mammary glands requires the expression of chemokine receptor CCR10 and α4β1 integrin on IgA ASC, which can interact with chemokine ligand CCL28 (mucosa-associated epithelial chemokine) and vascular cell-adhesion molecule-1 (VCAM-1), respectively. In the mouse mammary glands, the IgA ASC and the amount of IgA increase dramatically when the lactation starts and they decline after weanling (Weisz-Carrington *et al*. 1977). At the late pregnancy and lactation stage, the expression of CCL28 and VCAM-1 increase in the mammary glands, and the interplay of CCR10 with CCL28 and α4β1 with VCAM-1 allow the IgA plasma cell to migrate to the mammary glands (Bourges *et al*. 2008). The blockage of either CCL28 or VCAM-1 with antibodies can significantly suppress the homing of IgA ASC to the mammary glands (Low *et al*. 2010; Meurens *et al*. 2006; Wilson & Butcher 2004). Additionally, most IgA ASC express chemokine receptor CCR10, but IgA ASC from CCR10-deficient mice do not efficiently accumulate in the lactating mammary glands and lead to a significant decrease in milk IgA and fecal IgA of neonatal mice (Morteau *et al*. 2008). IgA is secreted as a dimeric form in mucosal immunity, whereas monomeric IgA is the common form in the blood (Brandtzaeg 1983). Transcytosis of dimeric IgA through secretory epithelial cells into the mucosal surface is mediated by the polymeric immunoglobulin receptor (pIgR) (Johansen & Kaetzel. 2011).

Several factors affect the mucosal IgA induction in the intestine and mammary glands in mammals. Supplemental vitamin A and β-carotene enhance the immune system in neonates **(**Bendich 1989; Chew & Park 2004), and β-carotene deficient calves were found to have a higher incidence of diarrhea in the first week of life (Kume & Toharmat 2001). β-cryptoxanthin is rich in mandarin oranges in Japan, and

β-cryptoxanthin as well as β-carotene is a typical fat-soluble carotenoid and has a pro-vitamin A activity (Rühl 2007). Vitamin A metabolite, all-*trans* retinoic acid (RA), plays important roles in gut immunity and several effects of carotenoids are thought to be mediated by their metabolism to vitamin A and subsequent mediation of retinoic acid receptor (RAR) and retinoid X receptor (RXR) response pathways (Rühl 2007). In the previous studies (Nishiyama *et al.* 2011a,b), β-carotene supplementation at 30 and 50 mg/kg in the diet in maternal mice during pregnancy and lactation increased the numbers of IgA ASC in the mammary glands and ileum of lactating mice and enhanced IgA transfer from maternal milk to neonatal mice. β-cryptoxanthin and β-carotene are inversely associated with the change of radial bone mineral density in post-menopausal female subjects (Sugiura *et al.* 2012) and β-cryptoxanthin suppresses the adipogenesis of 3T3-L1 cells via RAR activation (Shirakura *et al.* 2011).

Vitamin E is abundant in vegetable oils and has immunomodulatory effects. Studies in animal models suggest that vitamin E deficiency is associated with impairments in cellular and humoral immunity (Maydani *et al*. 2005; Webb & Villamor 2007). Vitamin E supplementation in the diet stimulated the primary development and proliferation of antibody-producing cells in mice (Tengerdy *et al.* 1973) and acted as an immunomodulator of total and antigen-specific IgA antibodies in chickens (Muir *et al*. 2002). Reactive oxygen species (ROS) are highly reactive and associated with oxidative stress in the body, and ROS can destroy cellular membranes, cellular proteins and nucleic acids (Chew & Park 2004). Vitamin E acts as an antioxidant in cellular membranes or a free radical scavenger by blocking the peroxidation of polyunsaturated fatty acids (Maydani *et al*. 2005; Moriguchi *et al*. 1990). Phytoestrogens are plant-derived compounds that have similar chemical structures to endogenous

estrogens and the potential to mimic estrogen activity (Ososki & Kennelly 2003), and can compete with 17β-estradiol for binding to estrogen receptors (ERs) as both estrogen agonists and antagonists (Ososki & Kennelly 2003; Setchell 1998). Coumestrol is a phytoestrogen and has various physiological effects through ERs (Morito *et al.* 2002). However, the exact mechanism of β-cryptoxanthin, vitamin E and coumestrol for enhancing IgA transfer from mother to neonates is still unclear, although the supplementation of β-cryptoxanthin, vitamin E and coumestrol have been expected to enhance the immune response in neonatal mice.

The objective of this study is to evaluate the relationships between fat-soluble vitamins and colostral IgG or IgA production in Japanese Black multiparous cows and to clarify the effects of supplemental coumestrol, β-cryptoxanthin and α-tocopherol on the IgA production in lactating mice. In Chapter II, I investigated the relationships among IgG, IgA, β-carotene, vitamin A and α-tocopherol contents in colostrum of Japanese Black multiparous cows in order to evaluate the role of fat-soluble vitamins on colostral IgG and IgA production. In Chapter III, I analyzed the effects of coumestrol administration to maternal mice during pregnancy and lactation on the numbers of IgA ASC in the mammary glands and ileum, mRNA expression of IgA C-region, MAdCAM-1, VCAM-1 and pIgR in the mammary glands and IgA transfer from maternal milk to neonatal mice. In Chapter IV, I evaluated the effects of β-cryptoxanthin supplementation at 50 mg/kg in the diet on the numbers of IgA ASC in the small intestine and mammary glands of lactating mice and IgA transfer from maternal milk to neonatal mice. In Chapter V, I examined the effects of α-tocopherol supplementation at 120 mg/kg in the diet on the numbers of IgA ASC in the small

intestine and mammary glands of lactating mice and IgA transfer from maternal milk to neonatal mice.

Chapter II

Relationships between Immunoglobulin and Fat-soluble Vitamins in Colostrum of Japanese Black Multiparous Cows

INTRODUCTION

Ig antibodies are main immune components in colostrum and the most abundant Ig in bovine colostrum is IgG (Stelwagen *et al.* 2009). Compared with colostral IgG, colostral IgA in cows is very low (Ishikawa *et al*. 1992), but IgA is the most abundant Ig isotype in mucosal secretions and provides protection against microbial antigens at mucosal surfaces in guts (Fagarasan & Honjo 2003; Mora & von Andrian 2009). The previous study (Yasumatsuya *et al.* 2012) has shown that feeding whey protein is useful to enhance mucosal IgA induction in calves, because feeding whey protein increased fecal IgA in Japanese Black calves after 14 days of age.

The transfer of passive immunity reduces the incidence and severity of scours in calves, although the disease resistance acquired from colostral Ig is only temporary and scours are common at 5 days to 3 weeks of age in calves (Quigley & Drewry 1998). However, the improvement of colostral IgG and IgA in Japanese Black cows is needed for the appropriate calf health and immune system, because the lower transfer of IgG and IgA from colostrum to neonatal calves was found in Japanese Black calves at 2 days of age (Yasumatsuya *et al*. 2013).

Supplemental vitamin A and β-carotene enhance the immune system in neonates (Chew & Park 2004; Rühl 2007), and vitamin E deficiency is associated with the impairments in the cellular and humoral immunity (Maydani *et al*. 2005; Webb &

Villamor 2007). High-quality silages contain large amounts of β-carotene and vitamin E, and β-carotene is converted to vitamin A by enzymes in the intestinal mucosa of cows (Johansson *et al*. 2014). Dietary levels of β-carotene and vitamin A affected not only colostral vitamin contents in cows but also vitamin status in newborn calves (Kume & Toharmat 2001). Supplemental β-carotene drastically increased serum β-carotene in Japanese Black calves (Nishiyama *et al*. 2011a), but β-carotene deficient calves were found to have a higher incidence of diarrhea in the first week of life (Kume & Toharmat 2001). Supplemental β-carotene in maternal mice during pregnancy and lactation increased the numbers of IgA ASC in the mammary glands of lactating mice and enhanced IgA transfer from maternal milk to neonatal mice (Nishiyama *et al*. 2011a, b). These results indicate that supplemental fat-soluble vitamins have been expected to improve colostral Ig in cows and health status in neonatal calves. However, it is not clear whether colostral IgG and IgA in cows were affected by dietary fat-soluble vitamins.

The study in Chapter II was conducted to clarify the relationships among IgG, IgA, β-carotene, vitamin A and α-tocopherol contents in colostrum of Japanese Black multiparous cows in order to evaluate the role of fat-soluble vitamins on colostral IgG and IgA production.

MATERIALS AND METHODS

Animals and diets

This research was approved by the guide for the care and use of cows in accordance with “Regulation on Animal Experimentation at Kyoto University” (Animal

Research Committee, Kyoto University, revised 2007). Data from 19 Japanese Black multiparous cows kept at Kyoto University Livestock Farm (Kyotanba, Japan) were collected from August 2013 to February 2014. The parity of cows at parturition was 4.9 ± 2.0 (mean ± SD), ranging from 2 to 9, and body weight of cows at 10 days before the expected calving date was 575 ± 46 kg (mean ± SD), ranging from 478 to 650 kg. The cows were managed in paddocks during the dry period and an individual calving pen from 10 days before the expected calving date to parturition.

The cows were given 2 kg/day of wheat bran and appropriate amounts of Italian ryegrass or Sudangrass round baled silages to meet the TDN requirements of breeding cows (Agriculture, Forestry, and Fisheries Research Council Secretariat 2008), but synthetic vitamins did not offer to the cows. Italian ryegrass silages were produced from the first, second and third cuttings in 2012 and the first cutting in 2013, and Sudangrass silages were produced from the first and second cuttings in 2012. The wheat bran contained 0.07 mg/kg β-carotene and 31.3 mg/kg α-tocopherol, but vitamin contents in silages were not determined.

Sample collection and analyses

Body weights of calves were measured at birth. Samples of colostrum were taken from each udder of the cows by hand approximately within 1 h after parturition, and 100 mL colostrum were stored at -20°C for chemical analyses.

Colostral IgG and IgA contents were measured using the Bovine IgG and IgA ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, USA) and ELISA Starter Accessory Package (Bethyl Laboratories) according to the manufacturer's instructions, respectively. Briefly, ninety-six well immunoplates (Thermo Fisher Scientific, Waltham,

MA, USA) were coated with 100 μL of sheep anti-bovine IgA or IgG coating antibody (10 μg/mL) overnight at 4°C. The plates were blocked with TBS containing 0.05% (w/v) Tween 20 (TBST) for 30 min, and washed five times with TBST. Then 100 μL of diluted samples were added to the wells, and incubated at room temperature for 60 min. After washing five times with TBST, 100 μL diluted HRP conjugated sheep anti-bovine IgA or IgG detection antibody was added to each well, and incubated for 60 min. After washing five times with TBST, 100 μL of TMB substrate solution was added to each well. After 15 min, the reaction was stopped with 2 M of sulfuric acid and the absorbance was measured at 450 nm immediately with a Microplate Reader (Multiskan FC; Thermo Fisher Scientific).

Colostral β-carotene, vitamin A and α-tocopherol were determined by high-performance liquid chromatography (HPLC). Briefly, 50-300 μL colostrum were homogenized in 3 mL of ethanol with 1 (vitamin A) or 6 (β-carotene and α-tocopherol) % pyrogallol. The homogenate was mixed with 300 μL of 60% KOH and heated for saponification. After the addition of 3 mL of water, vitamins were extracted with 6 mL of n-hexane and the extract was evaporated to dryness. The residue was dissolved in 150-500 μL of ethanol/chloroform (19:1) solution and 20 μL of the sample was subjected to HPLC equipped with Shimadzu (Kyoto, Japan) LC-10 AT pump and SPD-10A UV-VIS detector set at 480 (β-carotene), 325 (vitamin A), and 292 (α-tocopherol) nm absorbance, respectively. The combinations of mobile phase, column, and column temperature were methanol:tetrahydrofuran:water (94:5:1 v/v)/Vydac 201TP54 (Grace, Columbia, MD, USA)/21-24°C for β-carotene, methanol:water (92:8)/Shim-pack CLC-ODS(M) (Shimadzu)/40°C for vitamin A, and methanol (100)/Shim-pack CLC-ODS(M)/40°C for α-tocopherol, respectively. The flow rate of

mobile phase was 0.8 mL/min.

Statistical analysis

Relationships between Ig and fat-soluble vitamins in colostrum or other variables were examined by correlation and regression analyses of Statistical Analysis Systems (SAS 1997). Significance was declared at $P<0.05$.

RESULTS

The dystocia occurred in one cow, but no metabolic disorders were detected in the other cows and the health status of their calves was good at birth. The birth weight of calves was 34.3 ± 3.8 kg (mean ± SD), ranging from 28.0 to 41.0 kg, and the calf birth weight was positively correlated ($r = 0.59$; $P<0.01$) with the gestation length of dams.

Mean colostral IgG in cows was 141 mg/mL, ranging from 65 to 208 mg/mL, whereas mean colostral IgA was 8.7 mg/mL, ranging from 1.0 to 34.6 mg/mL (Table 2-1). Colostral IgG was positively correlated ($P<0.01$) with the age of cows, but colostral IgG was not correlated with the gestation length. There were positive correlations between colostral IgG and colostral vitamin A ($P<0.01$) or colostral α-tocopherol ($P<0.05$) in cows, but there were no relationships between colostral IgG and colostral IgA or colostral β-carotene (Fig. 2-1). The regression equations of age (X_{Age}), colostral vitamin A (X_{VA}) and colostral α-tocopherol (X_{VE}) on colostral IgG (Y_{IG}) were as follows.

$Y_{IG} = 0.928(\pm 0.245)^{**} X_{Age} + 73.6(\pm 18.7)^{**}$ ($R^2 = 0.43$, ** $P<0.01$)

$Y_{IG} = 0.082(\pm 0.023)^{**} X_{VA} + 101(\pm 12)^{***}$ ($R^2 = 0.40$, ** $P<0.01$, *** $P<0.001$)

$Y_{IG} = 0.031(\pm 0.012)^{*}X_{VE} + 111(\pm 13)^{***}$ ($R^2 = 0.24$, $^{*}P<0.05$, $^{***}P<0.001$)

There were no relationships between colostral IgA and fat-soluble vitamins in cows (Fig. 2-2). Colostral β-carotene was positively correlated with colostral vitamin A (r = 0.69; $P<0.001$) and colostral α-tocopherol (r = 0.63; $P<0.01$), and colostral vitamin A was positively correlated (r = 0.78; $P<0.001$) with colostral α-tocopherol. The regression equations of colostral β-carotene (X_{BC}) on vitamin A (Y_{VA}) and colostral α-tocopherol (Y_{VE}) and the regression equation of colostral vitamin A (X_{VA}) on colostral α-tocopherol (Y_{VE}) were as follows.

$Y_{VA} = 3.23(\pm 0.82)^{***}X_{BC} + 85(\pm 112)$ ($R^2 = 0.45$, $^{***}P<0.001$)

$Y_{VE} = 6.33(\pm 1.87)^{**}X_{BC} + 168(\pm 256)$ ($R^2 = 0.37$, $^{**}P<0.01$)

$Y_{VE} = 1.68(\pm 0.32)^{***}X_{VA} + 141(\pm 177)$ ($R^2 = 0.59$, $^{***}P<0.001$)

There was no relationship between colostral vitamin A and age of cows, but a highly significant relationship was obtained between colostral IgG (Y_{IG}) and age (X_{Age}) and colostral vitamin A (X_{VA}).

$Y_{IG} = 0.664(\pm 0.232)^{**}X_{Age} + 0.056(\pm 0.021)^{**}X_{VA} + 65.1(\pm 16.3)^{***}$ ($R^2 = 0.58$, $^{**}P<0.01$, $^{***}P<0.001$)

DISCUSSION

Colostrum contains not only nutrients but also biologically active substances that are essential for proper calf nutrition and health (Blum 2006; Stelwagen *et al.* 2009). The importance of adequate consumption of high-quality colostrum for acquisition of optimal nutrition and passive immunity is widely recognised in neonatal calves (Quigley & Drewry 1998; Stelwagen *et al.* 2009). The increased transfer of IgG and IgA

from maternal milk to neonates is needed for maintaining normal health in calves, but serum IgG and IgA were not detectable in colostrum-deprived calves at less than 2 days of age (Nonnecke *et al.* 2012). Additionally, colostral IgG contents as well as colostral protein of primiparous cows were lower than those of multiparous cows (Devery-Pocius & Larson 1983; Kume & Tanabe 1993a). The bovine mammary gland plays an active role in regulating Ig concentrations in colostrum, and immune factors in colostrum play critical roles in the host defense of the mammary glands, because the mammary gland is very susceptible to infection (Stelwagen *et al.* 2009). In the present study, colostral IgG was increased with aging in Japanese Black multiparous cows. Thus, the aging in multiparous cows may be a factor altering colostral IgG owing to the protection of the mammary gland from pathogenic organisms.

The absorption of colostral Ig by neonatal calves is considered adequate when serum IgG concentrations exceed 10 mg/mL, because the mortality rates of calves with serum IgG < 10 mg/mL were over twice than those of calves with higher IgG contents (Quigley & Drewry 1998). On the other hand, passive immune protection of the newborn gastrointestinal tract is dependent on an active process of IgA ASC accumulation in the lactating mammary glands of the mother, and IgA antibodies produced by IgA ASC in the mammary glands are secreted into milk (Morteau *et al.* 2008; Nishiyama *et al.* 2011b; Wang *et al.* 2013). Mean colostral IgG and IgA contents in Japanese Black cows in the present study agreed with those of the other report (Ishikawa *et al.* 1992), but colostral IgG and IgA varied widely and colostral IgG was not correlated with colostral IgA. The previous study (Yasumatsuya *et al.* 2013) has shown that there was no relationship between serum IgG and fecal IgA in Japanese Black calves at 2 days of age. These results suggest that the improving methods on IgG

and IgA production in colostrum are different in cows, although the adequate supply of IgG and IgA from colostrum is essential to gain sufficient passive immunity in neonatal calves.

The composition of colostrum varied with a number of factors, including individuality, breed, parity, prepartum diet and occurrence of mastitis, and the production and composition of colostrum varied with the uptake of nutrients by the mammary gland, which was influenced by mammary blood flow and utilization of nutrients in the mammary gland (Foley & Otterby 1978). In basic dairy cow diets, provitamin A (β-carotene) and vitamin E are mainly found in pasture and in grass and legume silages, but the contents were highly variable and synthetic vitamins are often supplemented in the diet (Johansson *et al.* 2014). As a result, colostral β-carotene in 46 Holstein cows was 169 ± 85 μg/dL (mean ± SD), ranging from 17.8 to 342.9 μg/dL, and colostral vitamin A was 122 ± 77 μg/dL (mean ± SD), ranging from 32.9 to 450.0 μg/dL (Kume & Toharmat 2001). Additionally, colostral β-carotene and vitamin A in Holstein multiparous cows were not affected by the parity, but colostral vitamin A in primiparous cows was high level (Kume & Tanabe 1993b). The wide range of colostral β-carotene and vitamin A in dairy cows may be due to the variable dietary levels of β-carotene and vitamin A and their uptake by the mammary glands (Kume & Tanabe 1993b; Kume & Toharmat 2001). In the present study, β-carotene, vitamin A and α-tocopherol contents in colostrum of Japanese Black multiparous cows varied widely, but highly positive correlations were obtained among β-carotene, vitamin A and α-tocopherol contents in colostrum. Thus, β-carotene, vitamin A and α-tocopherol contents in colostrum of cows may be changed in similar patterns owing to the quality of silages and their uptake by the mammary glands.

Several effects of carotenoids are thought to be mediated by their metabolism to vitamin A and subsequent mediation of RAR and RXR response pathways (Rühl 2007). β-carotene supplementation is effective to enhance mucosal IgA induction in the jejunum or ileum in weanling mice, and these effects may be mainly due to the RA-mediated immune response (Nishida *et al.* 2014). In the present study, high colostral vitamin A was related with high colostral IgG in Japanese Black cows, and the higher adjusted R^2 was obtained in the prediction model of colostral IgG from age and colostral vitamin A. Additionally, colostral α-tocopherol was positively correlated with colostral IgG, but colostral fat-soluble vitamins were not related with colostral IgA. These results indicate that feeding high-quality silages in pregnant cows has been expected to improve not only colostral fat-soluble vitamins but also colostral IgG contents. However, it is not clear whether supplemental vitamin A or β-carotene increases colostral IgG contents in cows owing to the RA-mediated immune response. Further study is needed to clarify the effects of fat-soluble vitamins on the passive immunity in calves, because a higher incidence of diarrhea occurred in the β-carotene deficient calves at 6 days of age (Kume & Toharmat 2001).

Table 2-1. Correlation between IgG, IgA and fat-soluble vitamins in colostrum of Japanese Black multiparous cows (n = 19).

	Mean	SD	Min.	Max.	*r*	
					IgG	IgA
Age, months	72.7	23.5	34.0	118.3	0.68**	0.25
Gestation length, days	288	5	277	296	0.32	0.37
Colostrum						
β-carotene, μg/dL	126	56	40	221	0.35	-0.07
Vitamin A, μg/dL	490	259	69	1096	0.66**	-0.01
α-tocopherol, μg/dL	963	554	117	2606	0.53*	0.17
IgA, mg/mL	8.7	9.7	1.0	34.6	0.32	-
IgG, mg/mL	141	32	65	208	-	-

** $P<0.01$, * $P<0.05$

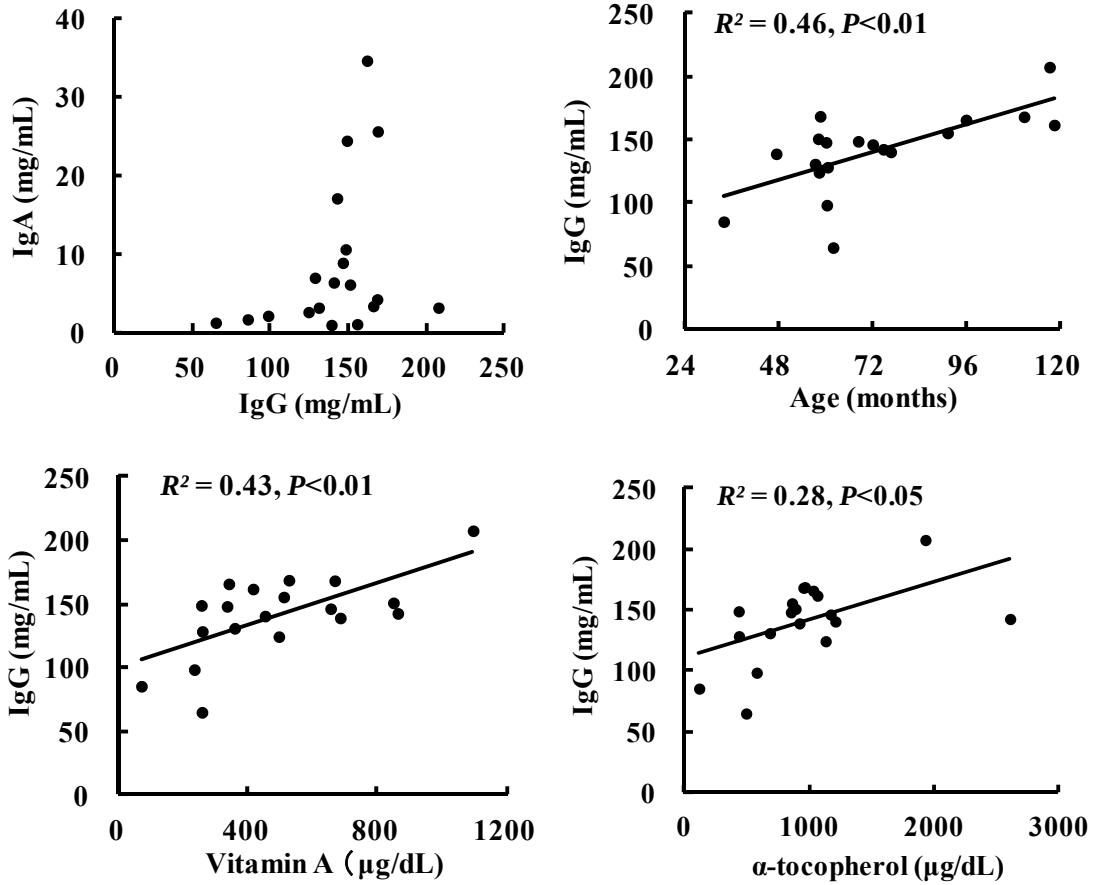


Figure 2-1. Relationships between colostral IgG and colostral IgA, vitamin A, α-tocopherol or age in Japanese Black multiparous cows (n = 19).

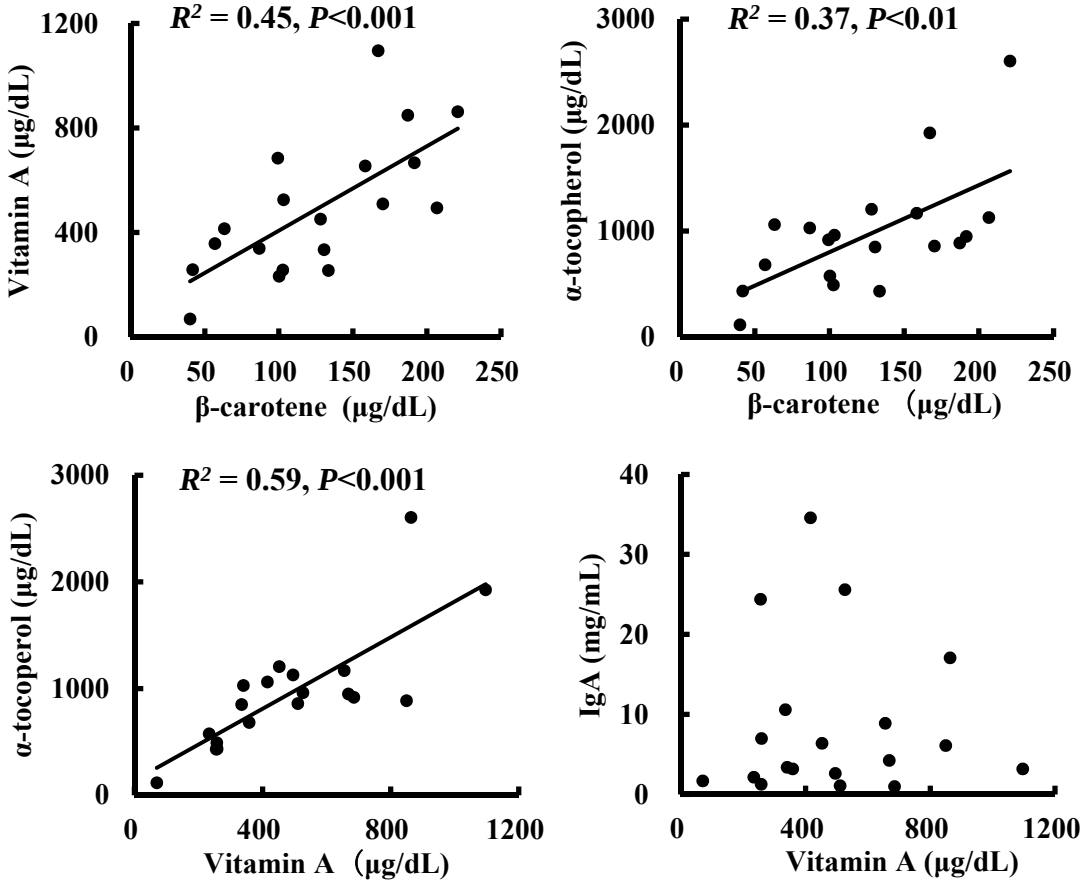


Figure 2-2. Relationships between colostral β-carotene and colostral vitamin A or α-tocopherol and relationships between colostral vitamin A and colostral α-tocopherol or IgA in Japanese Black multiparous cows (n = 19).

Chapter III

Effects of Coumestrol Administration to Maternal Mice during Pregnancy and Lactation on IgA-secreting Cells in Mammary Glands

INTRODUCTION

Coumestrol is a phytoestrogen and has various physiological effects through ERs (Morito *et al.* 2002). The previous studies (Kirihata *et al.* 2008, 2011; Ueda *et al.* 2012) indicated that the administration of coumestrol at a dose of 200 μg/kg body weight for pregnant and lactating mice, which is based on less than half the recommended threshold intake for isoflavones in the Ministry of Health, Labour and Welfare of Japan (Tokyo, Japan), decreased the activity of alkaline phosphatase in maternal duodenum around parturition and affected renal Ca metabolism in male neonatal mice at 11 days postpartum (dpp).

Mortality and morbidity of neonates continue to be major problems in humans and animals, and their most common disease is diarrhea. Passive immunity is critical to the survival and health of neonates, and colostrum or milk is a source of nutrients and immune components for neonates. IgA is the most abundant Ig isotype in mucosal secretions and provides protection against microbial antigens at mucosal surfaces (Fagarasan & Honjo 2003; Mora & von Andrian 2009). IgA antibodies produced by IgA ASC in mammary glands are secreted mainly as dimers after incorporation of the J chain and association with a transmembrane epithelial glycoprotein known as pIgR (Fagarasan & Honjo 2003). Because there might be significant changes in the ability to transport IgA from serum because of hormonal influences on the mammary tissue

(Halsey *et al.* 1982), supplemental phytoestrogen may be effective to enhance the immune system in humans and animals.

Passive immune protection of the newborn gastrointestinal tract is dependent on an active process of IgA ASC accumulation in lactating mammary glands of the mother, because IgA antibodies produced from IgA ASC in the mammary glands are secreted into milk (Morteau *et al.* 2008). IgA plasma cells in the mammary glands in mice are derived from lymphoid cells in the gut-associated lymphoid tissue by homing to the mammary gland (Roux *et al.* 1977). The efficient homing and accumulation of lymphocytes is highly dependent on cellular adhesion molecules expressed by the vascular endothelium and their integrin ligands, and the vasculature within the lactating mammary glands is known to express several adhesion molecules, including VCAM-1 and MAdCAM-1 (Low *et al.* 2010). In the previous studies (Nishiyama *et al.* 2011a, 2011b), supplemental β-carotene for maternal mice during pregnancy and lactation increased mRNA expression of IgA C-region and the numbers of IgA ASC in the ileum during lactation, but in the mammary glands, β-carotene supplementation only increased the numbers of IgA ASC. However, the mechanism of phytoestrogen for enhancing IgA ASC accumulation in the mammary glands of lactating animals is still unclear, although supplemental phytoestrogen has been expected to enhance ER-mediated immune response in the mammary glands.

The study in Chapter III was conducted to clarify the coumestrol administration to maternal mice during pregnancy and lactation on the numbers of IgA ASC in the mammary glands and ileum, mRNA expression of IgA C-region, MAdCAM-1, VCAM-1 and pIgR in the mammary glands and IgA transfer from maternal milk to neonatal mice.

MATERIALS AND METHODS

Animals and coumestrol treatment

Pregnant ICR mice were obtained from Clea Japan (Tokyo, Japan). They were housed in polycarbonate cages and maintained in an air-conditioned room (24±2°C) under controlled lighting conditions (light-dark cycle, 14:10 h). All mice were allowed free access to water and rodent feed (Oriental yeast, Tokyo, Japan). They received humane care as treated in accordance with 'Regulation on Animal Experimentation at Kyoto University' (Animal Research Committee, Kyoto University, revised 2007).

From 6.5 to 16.5 days postcoitus and 1 to 13 dpp, pregnant mice were administered a daily dose of coumestrol (200 μg/kg body weight/day, oral gavage, 23 times; Toronto Research Chemicals, Toronto, ON, Canada) dissolved in ethanol (Wako Pure Chemicals, Osaka, Japan) and purified olive oil (Wako; CM group, n = 7) or ethanol dissolved in purified olive oil (5 mL/kg/day) as vehicle control (VC group, n = 8) at 12.00 to 13.00 hours. As a normal control (NC group, n = 8), pregnant females were kept under the same conditions without administration of either vehicle or coumestrol solution. According to the previous studies (Nishiyama *et al.* 2011a, b), all the neonatal mice were alive by 7 dpp, and the numbers of pups for each mother were reduced to five female and five male neonatal mice at 7 dpp. All neonatal and maternal mice were sacrificed at 14 dpp.

Sample collection

Blood samples from maternal mice at 14 dpp were obtained by cardiac puncture

under anesthesia with Avertin (2, 2, 2-tribromoethanol, Sigma-Aldrich Chemical, MO, USA), and then mammary gland and ileum were removed, immediately frozen in dry ice-cooled isopentane (2-methylbutane, Wako Pure Chemicals, Osaka, Japan) for immunohistochemical analysis or frozen in liquid nitrogen and stored at -80°C for the semi-quantitative RT-PCR analysis. Blood samples from neonatal mice at 14 dpp were obtained by incising their hearts and collected with hematocrit tubes under anesthesia with Avertin, and then small intestine, stomach contents and rectum feces were rapidly removed. According to the previous studies (Nishiyama *et al.* 2011a, b), IgA concentration in neonatal stomach contents was represented as milk IgA level. Stomach contents and rectum feces were separately pooled to each mother and stored at -20°C, and the samples of small intestines were frozen in liquid nitrogen and stored at -80°C until IgA analysis. Blood samples from maternal or neonatal mice were left to stand at room temperature for 30 min and then centrifuged at 3000 rpm for 15 min or 10000 rpm for 5 min, respectively. Serum was fractionated for IgA analysis.

IgA immunoassay

Stomach contents and rectum feces were thawed, strongly vortexed in cold PBS containing protease inhibitor (Complete Mini; Roche, Basel, Switzerland) and centrifuged at 15000 rpm for 15 min at 4°C. Small intestine was homogenised using Sample Grinding Kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions and centrifuged at 15000 rpm for 10 min at 4°C. Each supernatant was fractionated for IgA analysis.

IgA concentrations in serum, stomach contents, small intestines and rectum feces were measured using the Mouse IgA ELISA Quantitation Kit (Bethyl Laboratories) and

ELISA Starter Accessory Package (Bethyl Laboratories) according to the manufacturer's instructions. Briefly, ninety-six well plates were coated with 100 μL of goat anti-mouse IgA (10 μg/mL) overnight at 4°C. The plates were blocked with 1% BSA in TBS for 30 min, and washed three times with TBS containing 0.05% (w/v) Tween 20 (TBST). Then 100 μL of diluted samples were added to the wells, and incubated for 60 min at room temperature. After washing five times with TBST, 100 μL diluted (1:30000) HRP conjugated goat anti-mouse IgA detection antibody was added to each well, and incubated for 60 min at room temperature. After washing five times with TBST, 100 μL of TMB substrate solution was added to each well. After 15 min the reaction was stopped with 2 M of sulfuric acid and the absorbance was measured at 450 nm immediately with a Microplate Reader (Multiskan FC; Thermo Fisher Scientific).

Immunohistochemical analysis

Fresh frozen sections (6 μm thick) mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Aldrich Chemical, Milwaukee, WI, USA) were fixed in 10 % neutral-buffered formalin (Wako Pure Chemicals) for 10 min at room temperature. After washing in PBS (pH 7·4), the sections were incubated with 1 % bovine serum albumin and 5 % normal donkey serum in PBS containing 5% (w/v) skimmed milk (blocking solution) for 20 min, successively with rabbit anti-mouse IgA (1:400 in the blocking solution; Open Biosystems, Huntsville, AL, USA) for 2 h at room temperature. After washing in PBS containing 0·02 % Tween 20 (PBST), the sections were incubated with Alexa fluor 555 donkey anti-rabbit IgG (1:500 in the blocking solution; Life Technologies, Carlsbad, CA, USA) for 90 min at room temperature. After washing in PBST, the sections were incubated with SYTOX green nucleic acid stain (1:20000

diluted in PBS; Life technologies) for 10 min. Then the sections were mounted with glycerol (Wako Pure Chemicals) and examined under an epifluorescence microscope (BX50, Olympus, Tokyo, Japan). The resulting images were analyzed by Image J software (National Institute of Health, Bethesda, MD, USA).

The IgA-positive cells in the mammary glands were counted in five randomised fields from each mouse and represented as IgA ASC/field of view (field = 1160 μm × 870 μm). Those in the ileum were counted in lamina propria of villi in five randomised villi from each mouse and represented as IgA ASC/unit area of lamina propria of villi (unit = 10000 μm^2).

Semi-quantitative RT-PCR

The mRNA expression of IgA C-region, MAdCAM-1 and VCAM-1 in the tissue was examined by semi-quantitative RT-PCR. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Maryland, CA, USA). Complementary DNA was synthesized with oligo-dT primer using a SuperScriptIII First-Strand Synthesis System for RT-PCR (Life technologies) from 4 μg RNA of each sample. The PCR was performed using Pt PCR SuperMix Kit (Life technologies). The PCR products were electrophoresed in 2% agarose gel and stained with 1 μg/mL ethidium bromide solution. After electrophoresis, the gels were recorded with a digital recorder and then mRNA expression levels were semi-quantified using Image J software. The relative abundance of specific mRNA was normalised by the abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

The primer pairs for IgA C-region were as follows: forward: 5'-TGCAC AGTTA CCCAT CCTGA-3', reverse: 5'-GCACCA GCACT TCTTT AGGG-3'. The PCR

procedure was as follows: after 95°C for 7 min to denature DNA, PCR was performed for twenty-seven cycles in the mammary glands or twenty-nine cycles in the jejunum and ileum at 95°C for 1 min, 53°C for 1 min, 72°C for 1 min, then at 72°C for 5 min. The primer pairs for MAdCAM-1 were as follows: forward: 5'-CCAGG CAGCA GTATC CTCTC-3', reverse: 5'-GAGAG ACTGT TCGGG TCTGC-3'. PCR cycles were as follows: after 95°C for 7 min to denature DNA, PCR performed for thirty-two cycles at 95°C for 1 min, 53°C for 1 min, 72°C for 1 min, then at 72°C for 5 min. The primer pairs for VCAM-1 were as follows: forward: 5'-ATTTT CTGGG GCAGG AAGTT-3', reverse: 5'-ACGTC AGAAC AACCG AATCC-3. PCR cycles were as follows: after 95°C for 7 min to denature DNA, PCR performed for thirty-four cycles at 95°C for 1 min, 53°C for 1 min, 72°C for 1 min, then at 72°C for 5 min. The primer pairs for pIgR were as follows: forward: 5'-GCTCC AAAGT GCTGT TCTCC-3', reverse: 5'-TTGCT GTGTG TCTGG AGAGG-3'. PCR cycles were as follows: after 95°C for 7 min to denature DNA, PCR performed for thirty-four cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, then at 72°C for 5 min. The primer pairs for GAPDH were as follows: forward: 5'-GGGTG GAGCC AAACG GGTC-3', reverse: 5'-GGAGT TGCTG TTGAA GTCGC-3'. The PCR procedure was as follows: after 95°C for 7 min to denature DNA, PCR was performed for twenty-five cycles in the mammary glands or twenty-seven cycles in the jejunum and ileum at 95°C for 1 min, 59°C for 1 min and 72°C for 1 min, then at 72°C for 5 min.

Statistical analysis

The general linear model procedure of Statistical Analysis Systems (SAS 1997) was used to analyse the effects of treatment on some variables in maternal mice and the

effects of treatment, sex and their interaction on some variables in neonatal mice. The differences were tested by Tukey-Kramer's multiple comparisons. Significance was declared at $P<0.05$, and trends were taken as $P<0.10$.

RESULTS

IgA concentration in serum and tissues

Body weight gains of maternal and neonatal mice were similar among the groups (Figure 3-1). In maternal mice, serum IgA concentration of CM group at 14 dpp was significantly higher ($P<0.05$) than that of VC group and tended to be higher ($P<0.10$) than that of NC group (Table 3-1). In neonatal mice, IgA concentrations in stomach contents, serum, intestine and feces at 14 dpp were not affected by treatment and sex.

IgA antibody-secreting cells in tissues

The numbers of IgA ASC in the mammary glands of CM group at 14 dpp were significantly higher ($P<0.001$) than those of NC and VC groups, but the numbers of IgA ASC in ileum were not affected by treatment (Table 3-2).

Expression of mRNA in mammary gland

The mRNA expression of IgA C-region of CM group at 14 dpp was significantly higher ($P<0.05$) than that of NC group. The mRNA expression of VCAM-1 in the mammary glands of CM group was significantly higher ($P<0.01$) than that of NC group and tended to be higher ($P<0.10$) than that of VC group. The mRNA expressions of MAdCAM-1 and pIgR were not affected by treatment (Table 3-3).

DISCUSSION

Successful neonatal health depends on many factors related to management and nutrition, but the improvement of the immune system is required for preventing diarrhea. The importance of adequate consumption of high-quality colostrum or milk for acquisition of optimal nutrition and passive immunity is widely recognised in neonates (Blum 2006; Quigley & Drewry 1998; Wheeler *et al.* 2007). Passive immune protection of the newborn gastrointestinal tract is dependent on an active production and storage of IgA ASC in the lactating mammary gland of the mother (Morteau *et al.* 2008).

Coumestrol administration to maternal mice during pregnancy and lactation increased the numbers of IgA ASC in the mammary glands and serum IgA of the mother at 14 dpp in the present study, but the numbers of IgA ASC in the ileum was not affected by coumestrol administration. On the other hand, β-carotene supplementation may have a predominant effect on IgA production in the ileum, although supplemental β-carotene increased the numbers of IgA ASC in the mammary glands (Nishiyama *et al.* 2011a). The mammary gland is colonised primarily by IgA-containing B cells during lactation (Tanneau *et al.* 1999). The enhancement of milk IgA and pIgR levels in the mammary glands during lactation may be resulted from hormonal influences on the mammary tissues (Halsey *et al.* 1982; Rincheval-Arnold *et al.* 2002), and some effects of estrogen were related to protection against oxidative injury of blood vessels (Mori *et al.* 2004). Estradiol plays an important role in regulating uterine IgA, and the increase in the IgA level that occurs spontaneously during the estrus cycle is probably due to the action of estradiol in the uterus (Wira & Sandoe 1980). Most IgA in lactating mice is

derived from the serum during early lactation and IgA synthesis by mammary cells becomes most important during late lactation (Halsey *et al.* 1982). Thus, the role of coumestrol for lactating mice is highly related to the efficient IgA ASC accumulation in the mammary glands, and the effects may be mainly due to the ER-mediated immune response on the lactating mammary glands.

The gut-associated lymphoid tissue is the largest immunologic organ in the body. Plasmablasts differentiated by IgA^+ B cell home preferentially to gut lamina propria through thoracic duct and blood (Mora & von Andrian 2009; Sigmundsdottir & Butcher 2008). Maternal IgA ASC primed in the gut homes to the mammary glands during late pregnancy and lactation (Roux *et al.* 1977; Tanneau *et al.* 1999). Recent study (Low *et al.* 2010) showed that the vascular adhesion molecule VCAM-1 and the lymphocyte-expressed integrin α4 are essential to the efficient migration of IgA ASC to the lactating mammary gland, but MAdCAM-1 and α4β7 were not required for efficient IgA ASC accumulation. In the present study, coumestrol administration increased the mRNA expression of VCAM-1 and IgA C-region in the mammary glands of maternal mice during lactation, but the mRNA expression of MAdCAM-1 in the mammary glands was not affected by coumestrol administration. These results indicate that coumestrol administration in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the mammary glands owing to the activated mRNA expressions of IgA C-region and VCAM-1 in the mammary glands.

Most IgA in neonatal mice may be derived from milk IgA, because very few IgA ASC were detected in the jejunum and ileum of neonatal mice at 14 dpp (Nishiyama *et al.* 2011a). IgA antibodies in milk are specific for antigens of the intestinal microflora and act to limit penetration of commensal intestinal bacteria through the neonatal

intestinal epithelium (Roux *et al*. 1977; Harris *et al*. 2006). Most IgA ASC express chemokine receptor CCR10, but IgA ASC from CCR10-deficient mice do not efficiently accumulate in the lactating mammary gland and lead to the significant decrease of milk IgA and fecal IgA of neonatal mice (Morteau *et al.* 2008). Supplemental β-carotene in maternal mice during pregnancy and lactation is useful for enhancing IgA transfer from maternal milk to neonates owing to the increase of IgA ASC in the mammary glands and ileum during lactation (Nishiyama *et al*. 2011a, 2011b). In the present study, however, coumestrol administration had no effect on milk IgA and mRNA expressions of pIgR in the mammary glands. Transport of IgA from immune cells in the mammary glands into colostrum and milk was regulated by pIgR (Wheeler *et al.* 2007), and supplemental fructooligosaccharides increased IgA response and pIgR expression in the intestines of infant mice (Nakamura *et al.* 2004). These results imply that coumestrol administration may have little effect on IgA transfer from maternal milk to neonate, which may be partly due to the unchanged pIgR expression during lactation.

In conclusion, coumestrol administration in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the mammary glands during lactation. However, further study is needed to clarify the effects of coumestrol administration on passive immunity in neonates, because coumestrol administration had no clear effect on IgA transfer from maternal milk to neonates.

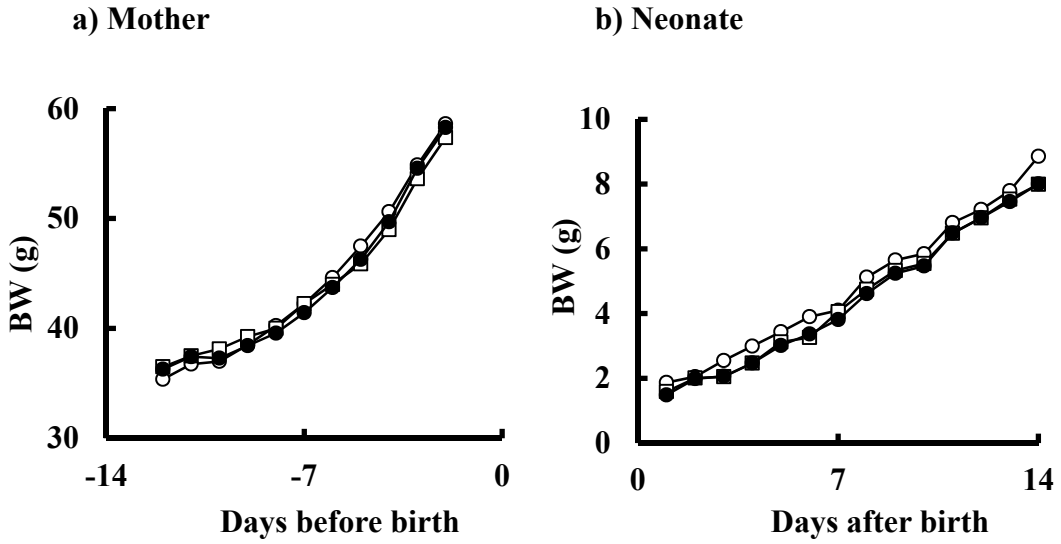


Figure 3-1. Body weight (BW) of maternal mice before parturition and neonatal mice after parturition in the control (○), vehicle control (□) and coumestrol (●) groups.

Table 3-1. IgA concentration in serum (μg/mL) of maternal mice and serum, stomach contents, small intestines and feces (μg/g) of neonatal mice in the control (NC), vehicle control (VC) and coumestrol (CM) groups at 14 days postpartum (Mean ± SE).

	NC	VC	CM	*P*
Mother				
Serum	480 ± 66 [ab]	430 ± 35 [a]	683 ± 55 [b]	*
Neonate				
Serum	2.3 ± 0.2	1.9 ± 0.2	2.3 ± 0.2	NS
Stomach contents	116 ± 15	118 ± 16	148 ± 8	NS
Intestine	173 ± 25	230 ± 48	236 ± 37	NS
Feces	2150 ± 384	2291 ± 307	2638 ± 300	NS

* $P<0.05$, [a, b] $P<0.05$

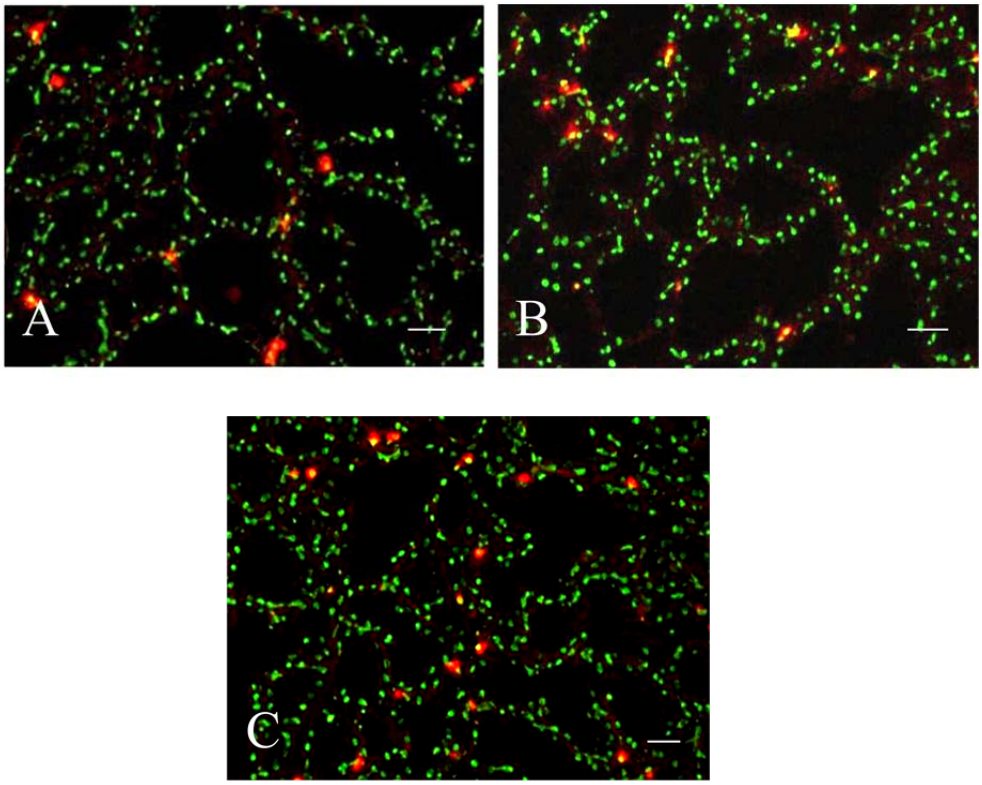


Figure 3-2. Immunofluorescent staining of IgA-secreting cells (ASC) in the mammary glands of lactating mice in the control (A), vehicle control (B) and coumestrol (C) groups at 14 days postpartum. IgA staining is shown in red, and nuclei are shown in green. Bar = 100 μm.

Table 3-2. Numbers of IgA ASC and the ratio of IgA C-region mRNA to GAPDH mRNA in the mammary glands and ileum of maternal mice in the control (NC), vehicle control (VC) and coumestrol (CM) groups at 14 days postpartum (Mean ± SD).

	NC	VC	CM	*P*
IgA ASC				
Mammary glands	6.9 ± 0.2 [a]	9.4 ± 0.2 [a]	15.5 ± 0.3 [b]	***
Ileum	4.1 ± 0.5	2.8 ± 0.8	3.4 ± 1.1	NS
IgA mRNA				
Mammary glands	0.83 ± 0.09 [c]	1.03 ± 0.09 [cd]	1.23 ± 0.06 [d]	*
Ileum	0.80 ± 0.06	0.63 ± 0.05	0.78 ± 0.06	NS

The numbers of IgA ASC in the mammary glands were counted in five randomized fileds from each mouse and represented as IgA ASC/field of view (field = 1160 μm × 870 μm). The numbers of IgA ASC in the ileum were counted in lamina propria of villi in five randomised villi from each mouse and represented as IgA ASC/unit area of lamina propria of villi (unit = 10000 μm^2).

*** $P<0.001$, * $P<0.05$, [a, b] $P<0.001$, [c, d] $P<0.05$

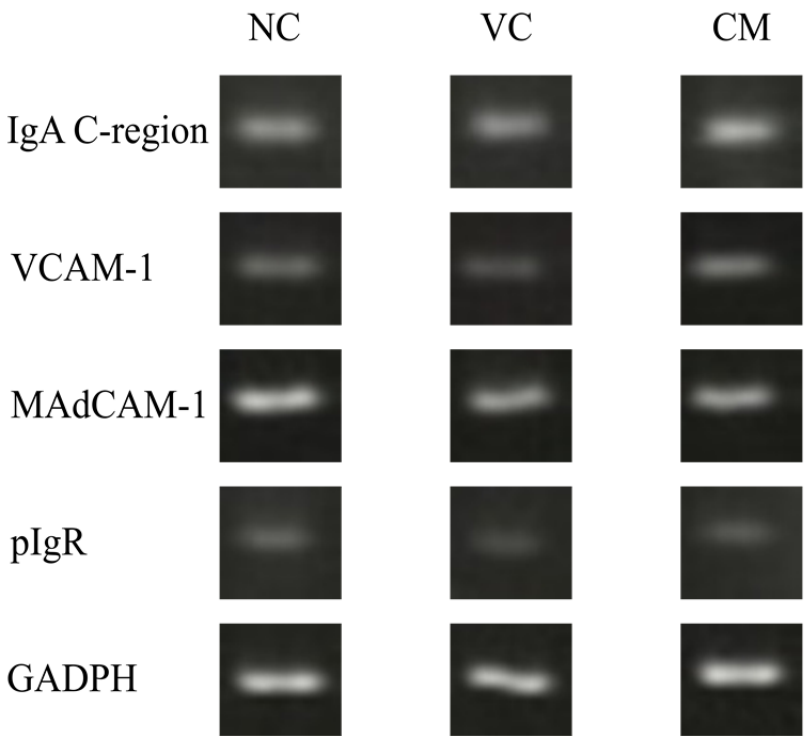


Figure 3-3. Expressions of IgA C-region, VCAM-1, MadCAM-1, pIgR and GAPDH mRNA in the mammary glands. VCAM-1, vascular cell adhesion molecule-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; pIgR, polymeric-Ig receptor; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

Table 3-3. The ratios of VCAM-1, MAdCAM-1 and pIgR mRNA to GAPDH mRNA in the mammary glands of maternal mice at 14 days postpartum (Mean ± SD).

	NC	VC	CM	*P*
VCAM-1	0.54 ± 0.05 [a]	0.76 ± 0.06 [ab]	0.92 ± 0.06 [b]	**
MAdCAM-1	0.79 ± 0.13	0.71 ± 0.09	0.68 ± 0.11	NS
pIgR	0.73 ± 0.05	0.89 ± 0.09	0.64 ± 0.12	NS

** $P<0.01$, [a, b] $P<0.01$

Chapter IV

Effects of Supplemental β-cryptoxanthin on IgA-secreting Cells in the Intestine and Mammary Glands of Lactating Mice

INTRODUCTION

Passive immunity is critical to the survival and health of neonates, and colostrum or milk is a source of nutrients and immune components for neonates. IgA is the most abundant Ig isotype in mucosal secretions and provides protection against microbial antigens at mucosal surfaces (Fagarasan & Honjo 2003; Mora & von Andrian 2009). Passive immune protection of the newborn gastrointestinal tract is dependent on an active process of IgA ASC accumulation in the lactating mammary glands of the mother, because IgA antibodies produced from IgA ASC in the mammary glands are secreted into milk (Morteau *et al.* 2008). β-cryptoxanthin is rich in mandarin orange in Japan, and β-cryptoxanthin as well as β-carotene is a typical fat-soluble carotenoid and has a pro-vitamin A activity (Rühl 2007).

Peyer's patches are the main site for the generation of IgA^+ B cells, and plasmablasts differentiated by IgA^+ B cells are preferentially homing on the gut lamina propria through the thoracic duct and blood by the expression of homing ligands and receptors (Ertesvåg *et al.* 2009; Fagarasan & Honjo 2003). The efficient homing and accumulation of lymphocytes is highly dependent on cellular adhesion molecules expressed by the vascular endothelium and their integrin ligands (Low *et al.* 2010). Coumestrol administration in maternal mice during pregnancy and lactation is effective

to increase the numbers of IgA ASC in the mammary glands during lactation owing to the activated mRNA expressions of IgA C-region and VCAM-1 in the mammary glands (Wang *et al.* 2013). On the other hand, chemokine ligand CCL25 is selectively expressed in the small intestine and CCL28 is widely expressed in the intestinal and nonintestinal mucosal tissues (Hieshima *et al.* 2004). Nishida *et al.* (2014) reported that β-carotene supplementation is effective to enhance the mucosal IgA induction in the jejunum of weanling mice owing to the increased mRNA expression of IgA C-region and CCL25. However, the mechanism of β-cryptoxanthin for enhancing mucosal immune induction in lactating animals is still unclear.

The study in Chapter IV was conducted to clarify the effects of β-cryptoxanthin supplementation at 50 mg/kg in the diet on the numbers of IgA ASC in the small intestine and mammary glands of lactating mice and IgA transfer from maternal milk to neonatal mice.

MATERIALS AND METHODS

Animals and diets

Pregnant ICR mice (n = 30) at 6.5 days postcoitus were purchased from Clea Japan (Tokyo, Japan). They were housed in polycarbonate cages and maintained in an air-conditioned room (24±2°C) under controlled lighting conditions (light-dark cycle, 14:10 h). They received humane care as treated in accordance with 'Regulation on Animal Experimentation at Kyoto University' (Animal Research Committee, Kyoto University, revised 2007).

Pregnant mice were randomly allocated to the control or β-cryptoxanthin group at 6.5 days postcoitus. Mice in the control group were fed rodent feed (Oriental Yeast, Tokyo, Japan) from 6.5 days postcoitus to 7 (n = 8) or 14 (n = 8) dpp, and those in the β-cryptoxanthin group were fed 50 mg/kg β-cryptoxanthin-supplemented rodent feed from 6.5 days postcoitus to 7 (n = 7) or 14 (n = 7) dpp. The rodent feed contained 55.3% NFE, 23.6% CP, 5.1% crude fat, 5.8% crude ash, 1283 IU/100g vitamin A and 9.1 mg/100g vitamin E. In the β- cryptoxanthin group, β-cryptoxanthin (Unitika Ltd., Uji, Japan) was mixed with the rodent feed at 50 mg/kg, which was similar to the dietary β-carotene level as previously described (Nishiyama *et al*. 2011a). All the neonatal mice were alive by 2 dpp, and the numbers of pups for each mother were reduced to five female and five male neonatal mice at 2 dpp. Then, five female and five male neonatal mice born to each mother and the maternal mice were dissected at 7 or 14 dpp.

All mice were allowed free access to water and feed. Body weights and feed intake of maternal mice and body weights of neonatal mice were measured at 12.00 hours every day

Sample collection

Blood samples from maternal mice of the control and β-cryptoxanthin groups were obtained by cardiac puncture under anaesthesia with Avertin (2, 2, 2-tribromoethanol, Sigma-Aldrich Chemical) at 7 and 14 dpp, and then mammary glands, jejunum and ileum were removed after euthanasia by cervical dislocation. The samples of mammary glands, jejunum and ileum were immediately frozen in dry ice-cooled isopentane (2-methylbutane, Wako Pure Chemicals) for

immunohistochemical analysis or frozen in liquid nitrogen and stored at -80°C for semi-quantitative RT-PCR.

Blood samples from neonatal mice at 7 or 14 dpp were obtained by incising their hearts and collecting with hematocrit tubes under anesthesia with Avertin, and then small intestines, stomach contents and rectum feces were rapidly removed. According to the previous studies (Nishiyama *et al.* 2011a, b), IgA concentrations in stomach contents were represented as milk IgA level. The samples were pooled for all neonatal mice born to each mother at 7 or 14 dpp. The samples of small intestines were frozen in liquid nitrogen and stored at -80°C and the samples of stomach contents and rectum feces were stored at -20°C.

Blood samples from maternal or neonatal mice were left to stand at room temperature for 1 h or 30 min and then centrifuged at 3000 rpm for 15 min or 10000 rpm for 5 min, respectively. The samples of serum were stored at -20°C.

IgA immunoassay and immunohistochemical analysis

IgA immunoassay of serum, stomach contents, small intestines and feces, and immunohistochemical analysis of the mammary glands, jejunum and ileum were determined as described in Chapter III. IgA concentrations in serum, stomach contents, small intestines and rectum feces were measured using the Mouse IgA ELISA Quantitation Kit (Bethyl Laboratories) and ELISA Starter Accessory Package (Bethyl Laboratories) according to the manufacturer's instructions.

The sections obtained from the immunohistochemical analysis were examined under an epifluorescence microscope (BX50, Olympus), and the resulting images were analyzed by Image J software. The IgA-positive cells in the mammary glands were

counted in eight randomised fields from each mouse and represented as IgA ASC/field of view (field = 1160 μm × 870 μm). Those in the jejunum and ileum were counted in lamina propria of villi in eight randomised villi from each mouse and represented as IgA ASC/unit area of lamina propria of villi (unit = 10000 μm^2).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as described in Chapter III. The mRNA expression of IgA C-region, CCL25 and CCL28 in the jejunum and ileum and the mRNA expression of IgA C-region, CCL28 and VCAM-1 in the mammary glands were examined. The primer pairs for CCL25 were as follows: forward: 5'-CCTTC AGGTA TCTGG AGAGG AGATC-3', reverse: 5'-CAAGA TTCTT ATCGC CCTCT TCA-3'. The PCR procedure was as follows: after 95°C for 5 min to denature DNA, PCR was performed for thirty cycles in the jejunum and ileum or thirty-six cycles in the mammary glands at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, then at 72°C for 7 min. The primer pairs for CCL28 were as follows: forward 5'-TGGCA AAAGC CACAT TCATA-3', reverse: 5'-CATGC CAGAG TCGAA CAGAA-3'. The PCR procedure was as follows: after 95°C for 5 min to denature DNA, PCR performed for forty-five cycles in the jejunum and ileum or thirty-seven cycles in the mammary glands at 95°C for 1 min, 53°C for 1 min, 72°C for 1 min, and then at 72°C for 7 min.

Statistical analysis

Data from bodyweight and feed intake of maternal mice and bodyweight of neonatal mice during prepartum or postpartum periods were analyzed by least squares ANOVA using the general linear models procedure of SAS (1997). The model was as follows:

$Y_{ijk} = \mu + T_i + M_{(i)j} + D_k + TD_{ik} + e_{ijk}$

where μ is the overall mean, T_i is the effect of treatment, $M_{(i)j}$ is the random variable of a mice nested in treatment, D_k is the effect of day, TD_{ik} is the interactions, and e_{ijk} is the residuals. The general linear model procedure of Statistical Analysis Systems (SAS 1997) was used to analyse the effects of treatment or time on some variables in maternal mice and neonatal mice. Significance was declared at $P<0.05$

RESULTS

IgA concentrations in serum and tissues

Bodyweight gains and feed intake of maternal mice during prepartum and 7 or 14 dpp were not affected by the treatment (Figure 4-1). Bodyweight gains of neonatal mice were not affected by the treatment (data not shown).

In neonatal nice, IgA concentrations in serum, stomach contents, small intestines and feces at 7 and 14 dpp were not affected by the treatment (Figure 4-2). Compared with IgA concentrations of neonatal mice at 7 dpp, IgA concentrations in serum ($P<0.001$), stomach contents ($P<0.001$) and feces ($P<0.05$) increased at 14 dpp. In maternal mice, serum IgA concentrations at 7 and 14 dpp were not affected by the treatment.

IgA antibody-secreting cells in tissues

In maternal mice, the numbers of IgA ASC in the jejunum of the β-cryptoxanthin group at 14 dpp were significantly higher ($P<0.05$) than those of the control group, but the numbers of IgA ASC in the ileum and the mammary glands were not affected by

the treatment (Table 4-1). Compared with the numbers of IgA ASC of maternal mice at 7 dpp, the numbers of IgA ASC in the mammary glands ($P<0.001$) and ileum ($P<0.01$) increased at 14 dpp.

Expression of mRNA in tissues

In maternal mice, the mRNA expressions of IgA C-region in the jejunum ($P<0.001$), ileum ($P<0.05$) and the mammary glands ($P<0.001$) of the β-cryptoxanthin group at 14 dpp were significantly higher than those of the control group (Table 4-1). The mRNA expressions of CCL25 ($P<0.05$) and CCL28 ($P<0.01$) in the jejunum of the β-cryptoxanthin group were significantly higher than those of the control group at 14 dpp, but the mRNA expressions of CCL25 and CCL28 in the ileum and VCAM-1 in the mammary glands were not affected by the treatment (Table 4-2).

DISCUSSION

The mammary glands of mice develop new vasculature during pregnancy, and it is colonized primarily by IgA-containing B cells during lactation (Halsey *et al*. 1982). Very few IgA ASC were detected in the mammary glands of maternal mice during pregnancy and the numbers of IgA ASC in the mammary glands increased at 14 dpp, but the numbers of IgA ASC in the jejunum and ileum were similar during pregnancy and lactation (Nishiyama *et al*. 2011a). In the present study, the numbers of IgA ASC in the mammary glands of maternal mice at 14 dpp were about 2 times higher than those at 7 dpp, but the numbers of IgA ASC in the jejunum were similar at 7 and 14 dpp. Relative IgA mRNA levels increased dramatically at birth and continued to increase

through the lactation period (Low *et al.* 2010), and IgA concentrations in stomach contents of neonatal mice, which represented milk IgA level, increased drastically with age in the previous (van der Feltz *et al*. 2001) and present study. These results indicated that the numbers of IgA ASC in the mammary glands of mice and IgA transfer from maternal mice to neonatal mice increased drastically with age during lactation, but the numbers of IgA ASC in the jejunum and ileum may be almost constant during pregnancy and lactation.

IgA plasma cells in the mammary glands in mice are derived from lymphoid cells in the gut-associated lymphoid tissue by homing to the mammary glands (Roux *et al.* 1977). In the previous study (Nishiyama *et al.* 2011a), supplemental β-carotene at 50 mg/kg in the diet in maternal mice during pregnancy and lactation increased the numbers of IgA ASC and mRNA expression of IgA C-region in the ileum during lactation, but in the mammary glands, β-carotene supplementation only increased the numbers of IgA ASC. On the other hand, supplemental β-cryptoxanthin at 50 mg/kg in the diet in maternal mice during pregnancy and lactation increased the numbers of IgA ASC and the mRNA expression of IgA C-region in the jejunum at 14 dpp in the present study. However, β-cryptoxanthin supplementation had no effect on the numbers of IgA ASC in the ileum and mammary glands, although supplemental β-cryptoxanthin increased the mRNA expression of IgA C-region in the ileum and mammary glands at 14dpp. These results imply that supplementation of β-cryptoxanthin in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the jejunum during late lactation.

CCL25 plays an essential role in intestinal homing of IgA ASC primarily by mediating their extravasation into intestinal lamina propria, and CCL28 is expressed in

the mucosal tissues of intestines and mammary glands (Hieshima *et al.* 2004). Supplemental β-carotene increased the mRNA expressions of CCL25 and IgA ASC in the jejunum of weanling mice after 14 and 21 days of treatment (Nishida *et al.* 2014). Because supplemental β-cryptoxanthin increased the mRNA expressions of CCL25 and CCL28 in the jejunum of maternal mice at 14 dpp in the present study, the increased IgA ASC in the jejunum of lactating mice caused by β-cryptoxanthin supplementation may be due to the increased mRNA expressions of CCL25 and CCL28 in the jejunum.

IgA antibodies in milk are specific for antigens of the intestinal microflora and act to limit penetration of commensal intestinal bacteria through the neonatal intestinal epithelium (Harris *et al.* 2006; Roux *et al.* 1977). Supplemental β-carotene at 30 and 50 mg/kg in the diet in maternal mice during pregnancy and lactation is useful for enhancing IgA transfer from maternal milk to neonates during lactation (Nishiyama *et al*. 2011a, b). However, supplemental β-cryptoxanthin had no effect on IgA concentrations in stomach contents of neonatal mice as well as the numbers of IgA ASC in the mammary glands in the present study. Thus, compared with β-carotene, supplemental β-cryptoxanthin may have little effect on IgA transfer from maternal milk to neonatal mice. However, the present study demonstrates that β-cryptoxanthin supplementation in maternal mice during pregnancy and lactation is effective to enhance mucosal IgA induction in the jejunum, because supplemental β-cryptoxanthin increased the numbers of IgA ASC and mRNA expressions of IgA C-region, CCL25 and CCL28 in the jejunum during late lactation.

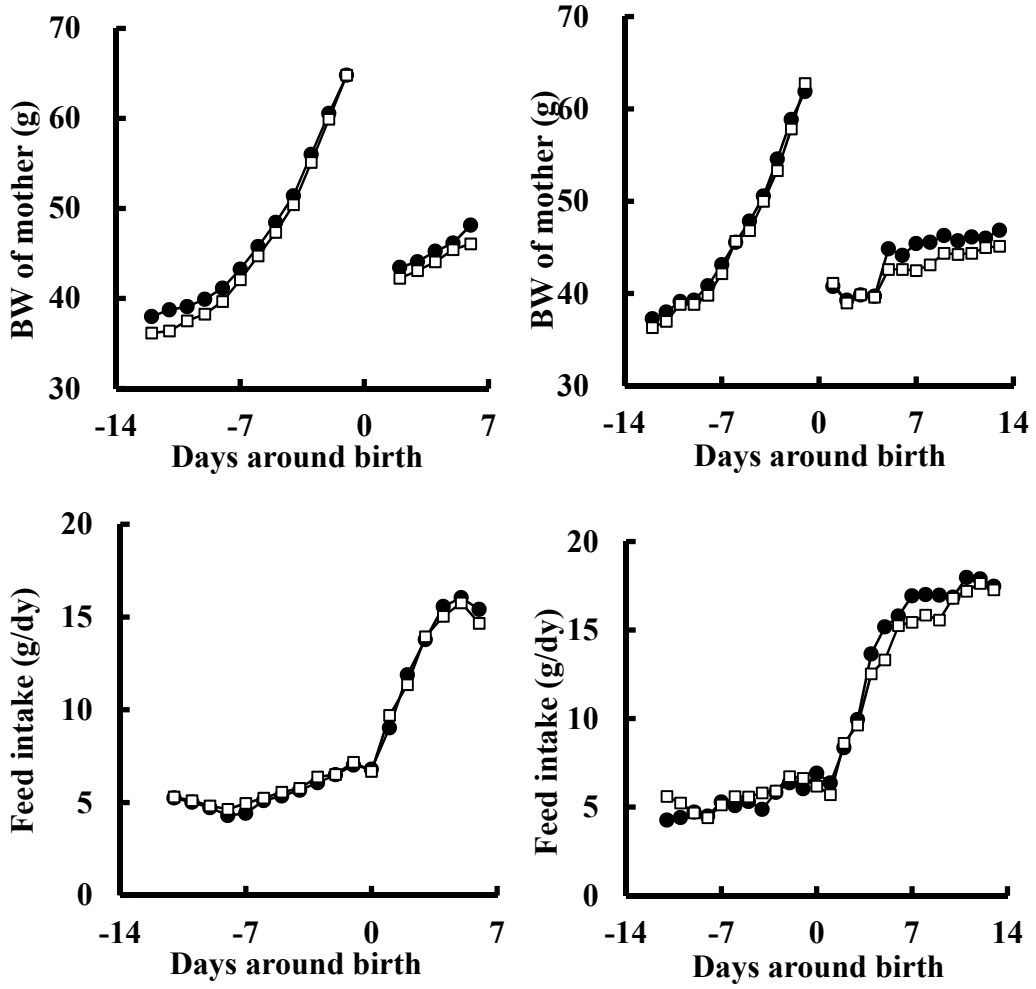
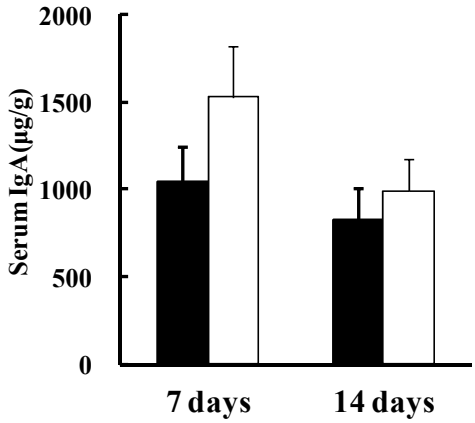


Figure 4-1. Bodyweight (BW) and feed intake of maternal mice around parturition of the control (□) and β-cryptoxanthin (●) groups during prepartum and 7 or 14 days postpartum.

(a) Lactating mice



(b) Neonatal mice

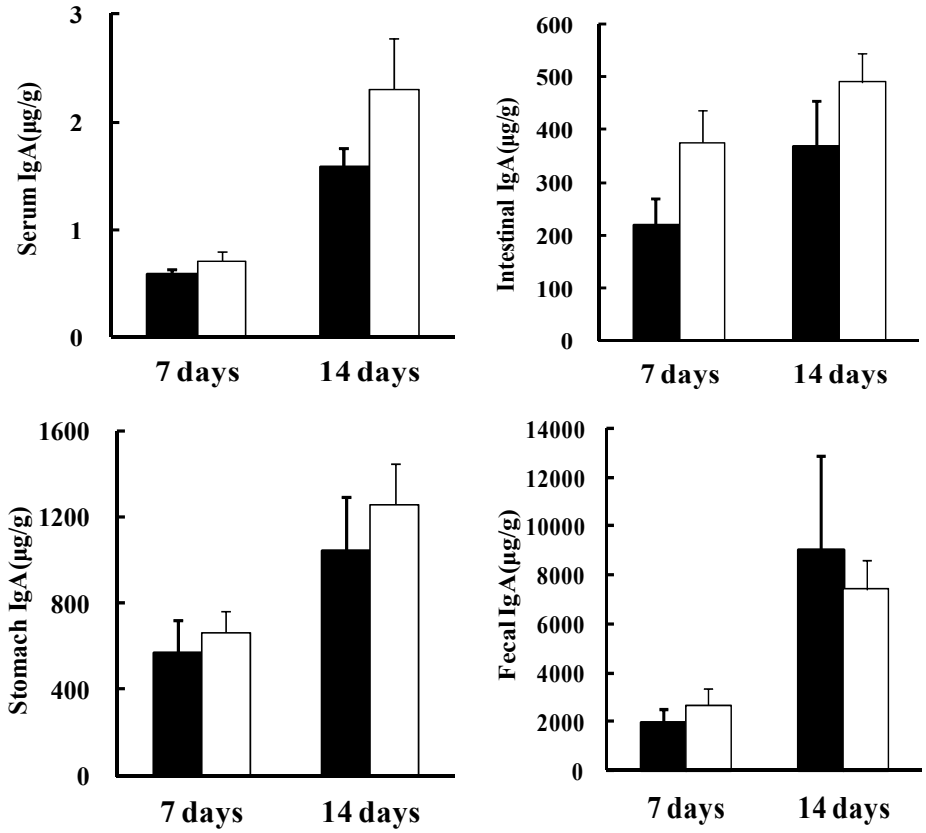


Figure 4-2. IgA concentration in serum of lactating mice (a), and serum, stomach contents, small intestines and feces of neonatal mice (b) of the control (■) and β-cryptoxanthin (□) groups at 7 and 14 days postpartum (Mean ± SE).

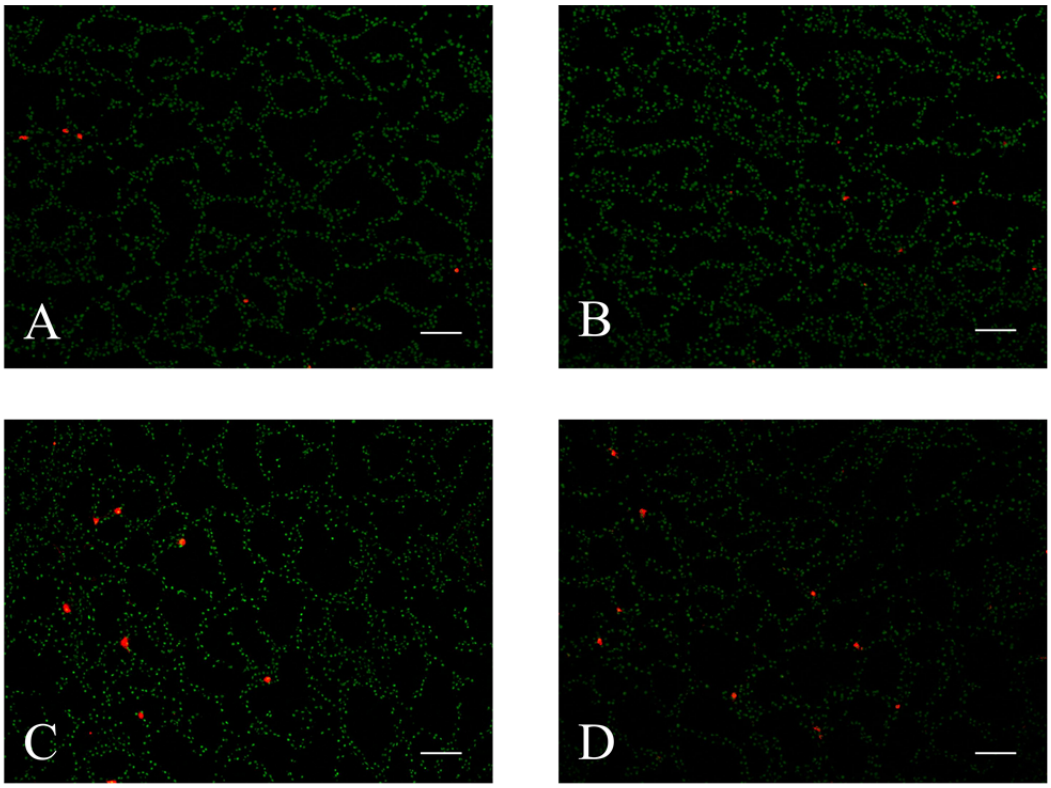


Figure 4-3. Immunofluorescent staining of IgA-secreting cells (ASC) in the mammary glands of lactating mice in the control (A) and β-cryptoxanthin (B) groups at 7 days postpartum, and in the control (C) and β-cryptoxanthin (D) groups at 14 days postpartum. IgA staining is shown in red, and nuclei are shown in green. Bar = 100 μm.

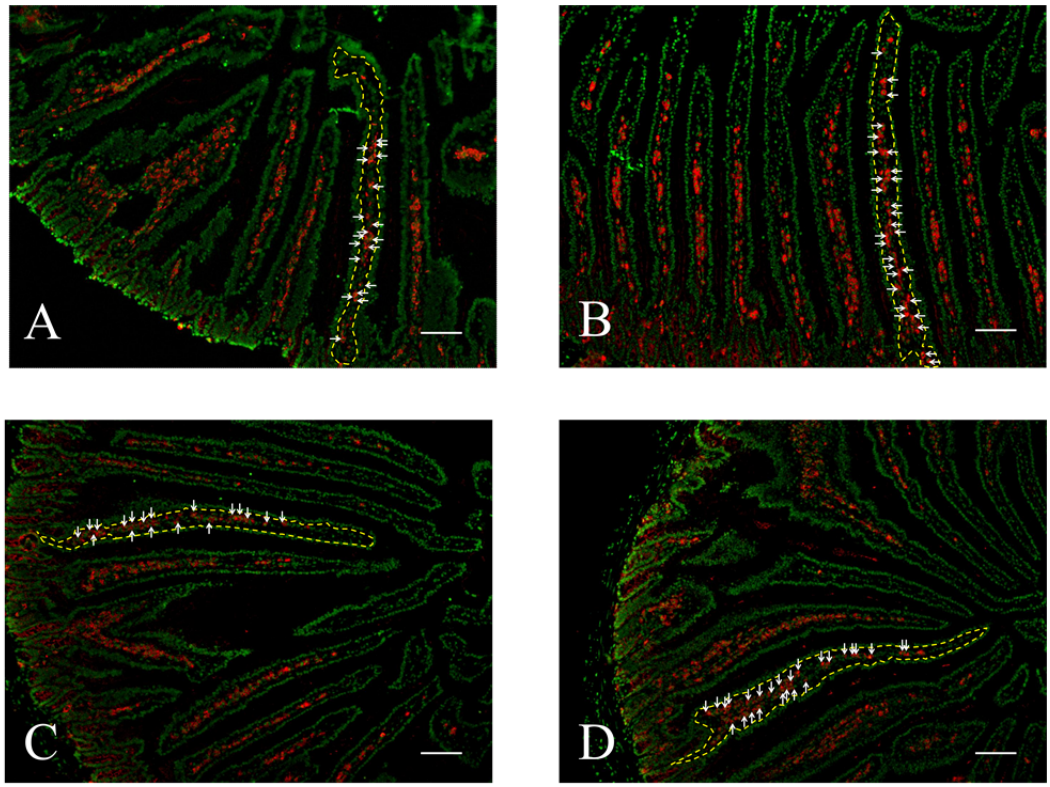


Figure 4-4. Immunofluorescent staining of IgA ASC in the jejunum of lactating mice in the control (A) and β-cryptoxanthin (B) groups at 7 days postpartum, and in the control (C) and β-cryptoxanthin (D) groups at 14 days postpartum. IgA staining is shown in red, and nuclei are shown in green. The enclosed area by yellow dashed line represents the lamina propria of villi, and arrows represent IgA ASC. Bar = 100 μm.

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Table 4-1. Numbers of IgA ASC and the ratios of IgA C-region mRNA to GAPDH mRNA in the mammary glands, jejunum and ileum of maternal mice in the control and β-cryptoxanthin groups at 7 and 14 days postpartum (Mean ± SD).

	Days	Control	β-cryptoxanthin	*P*
IgA ASC				
Mammary glands	7	3.9 ± 1.0	4.1 ± 0.9	NS
	14	6.8 ± 0.4	8.9 ± 0.9	NS
Jejunum	7	10.9 ± 0.3	12.5 ± 1.0	NS
	14	10.7 ± 0.7	13.1 ± 0.6	*
Ileum	7	10.5 ± 0.9	11.6 ± 0.8	NS
	14	12.8 ± 0.6	14.0 ± 0.7	NS
IgA mRNA				
Mammary glands	7	0.61 ± 0.11	0.89 ± 0.11	NS
	14	0.55 ± 0.08	1.11 ± 0.07	***
Jejunum	7	0.80 ± 0.10	0.97 ± 0.09	NS
	14	0.78 ± 0.09	1.43 ± 0.13	**
Ileum	7	0.78 ± 0.09	0.85 ± 0.04	NS
	14	0.53 ± 0.04	0.67 ± 0.05	*

*** $P<0.001$, ** $P<0.01$, * $P<0.05$

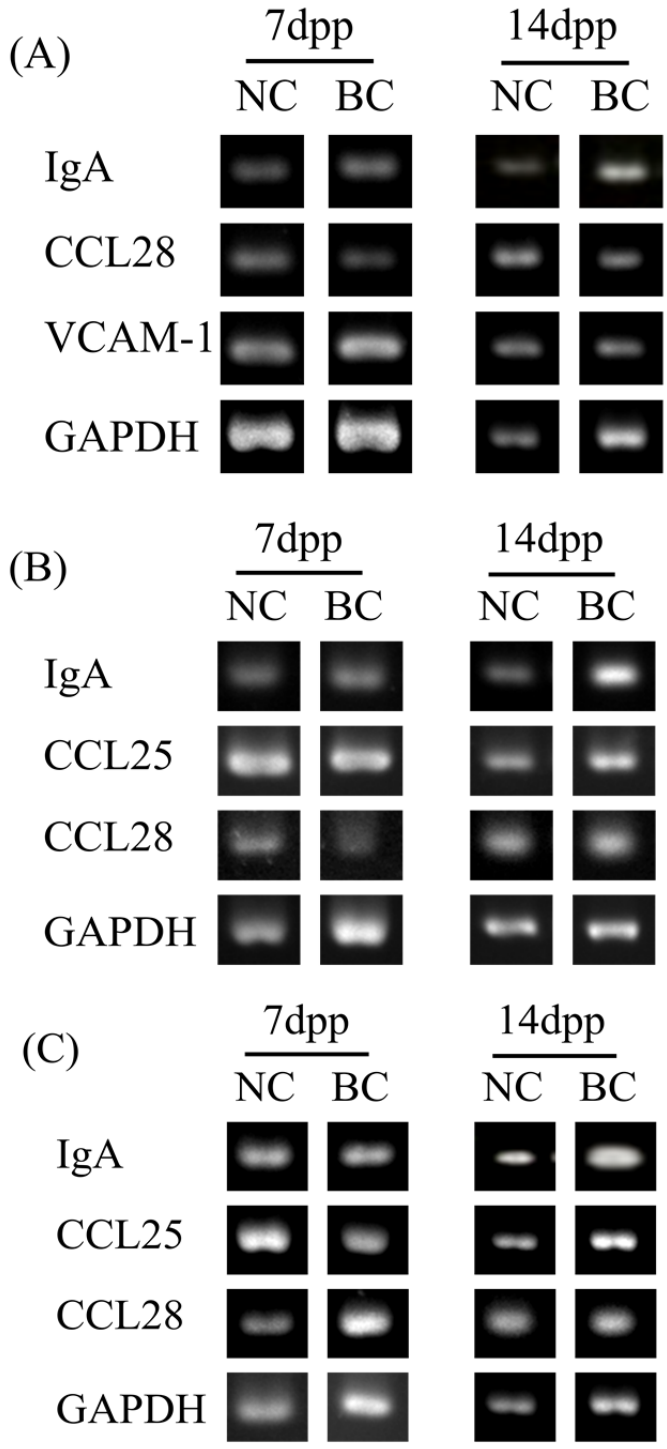


Figure 4-5. Expressions of IgA C-region, VCAM-1, CCL25, CCL28 and GAPDH mRNA in the mammary glands (A), jejunum (B) and ileum (C). VCAM-1, vascular cell adhesion molecule-1; CCL25, chemokine ligand 25; CCL28, chemokine ligand 28; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

Table 4-2. The ratios of IgA C-region, CCL25, CCL28 and VCAM-1 mRNA to GAPDH mRNA in the mammary glands, jejunum and ileum of maternal mice in the control and β-cryptoxanthin groups at 7 and 14 days postpartum (Mean ± SE).

	Days	Control	β-cryptoxanthin	*P*
Mammary glands				
CCL28	7	0.74 ± 0.13	0.73 ± 0.20	NS
	14	0.99 ± 0.13	1.18 ± 0.20	NS
VCAM-1	7	0.82 ± 0.08	0.70 ± 0.11	NS
	14	0.93 ± 0.15	0.93 ± 0.12	NS
Jejunum				
CCL25	7	1.16 ± 0.14	1.04 ± 0.08	NS
	14	0.50 ± 0.03	0.88 ± 0.15	*
CCL28	7	0.78 ± 0.16	0.79 ± 0.08	NS
	14	0.73 ± 0.04	1.42 ± 0.21	**
Ileum				
CCL25	7	1.11 ± 0.06	1.21 ± 0.05	NS
	14	1.29 ± 0.08	1.13 ± 0.07	NS
CCL28	7	0.68 ± 0.07	0.68 ± 0.03	NS
	14	1.01 ± 0.10	1.09 ± 0.11	NS

** $P<0.01$, * $P<0.05$

Chapter V

Effects of Supplemental α-tocopherol on IgA-secreting Cells in the Intestine and Mammary Glands of Lactating Mice

INTRODUCTION

Vitamin E is abundant in vegetable oils and has immunomodulatory effects, and studies in animal models suggest that vitamin E deficiency is associated with impairments in cellular and humoral immunity (Maydani *et al*. 2005; Webb & Villamor 2007). A number of endogenous antioxidants are produced in the body and can scavenge harmful ROS to maintain the normal cellular function and health, but dietary sources of antioxidants are required to eliminate excessive ROS in the body under conditions of high oxidative stress (Chew & Park 2004). β-carotene supplementation increased mRNA expression of IgA C-region and the numbers of IgA ASC in ileum of lactating mice, and also increased IgA transfer from maternal milk to neonates (Nishiyama *et al*. 2011a). However, the mechanism of α-tocopherol for enhancing mucosal immune induction in lactating animals is still unclear.

The study in Chapter V was conducted to clarify the effects of α-tocopherol supplementation at 120 mg/kg in the diet on the numbers of IgA ASC in the small intestine and mammary glands of lactating mice and IgA transfer from maternal milk to neonatal mice.

MATERIALS AND METHODS

Animals and diets

Pregnant ICR mice (n = 15) at 6.5 days postcoitus were purchased from Clea Japan (Tokyo, Japan). They were housed in individual polycarbonate cages and maintained in an air-conditioned room (24±2°C) under controlled lighting conditions (light-dark cycle, 14:10 h). They received humane care as treated in accordance with 'Regulation on Animal Experimentation at Kyoto University' (Animal Research Committee, Kyoto University, revised 2007).

Pregnant mice were randomly allocated to the control or α-tocopherol group at 6.5 days postcoitus. From 6.5 days postcoitus to 7 or 14 dpp, mice in the control group (n = 7) were fed rodent feed (Oriental Yeast, Tokyo, Japan) and those in the α-tocopherol group (n = 8) were fed 120 mg/kg α-tocopherol-supplemented rodent feed. The rodent feed contained 55.3% NFE, 23.6% CP, 5.1% crude fat, 5.8% crude ash, 1283 IU/100g vitamin A and 91 mg/kg vitamin E. In the α-tocopherol group, α-tocopherol (Mitsubishi Kagaku Foods Co. Ltd., Tokyo, Japan) was mixed with the rodent feed at 120 mg/kg. All the neonatal mice were alive by 2 dpp, and the numbers of pups for each mother were reduced to five female and five male neonatal mice at 2 dpp. Then, all neonatal mice and the maternal mice were dissected at 14 dpp.

All mice were allowed free access to water and feed. Body weights and feed intake of mice and body weights of neonatal mice were measured at 12.00 hours every day.

Sample collection

Blood samples from maternal mice of the control or α-tocopherol group were obtained by cardiac puncture under anaesthesia with Avertin (2, 2, 2-tribromoethanol, Sigma-Aldrich Chemical) at 14 dpp, and then mammary glands, jejunum and ileum

were removed after euthanasia by cervical dislocation. The samples of mammary glands, jejunum and ileum were immediately frozen in dry ice-cooled isopentane (2-methylbutane, Wako Pure Chemicals) for immunohistochemical analysis or frozen in liquid nitrogen and stored at -80°C for quantitative RT-PCR.

Blood samples from neonatal mice at 14 dpp were obtained by incising their hearts and collecting with hematocrit tubes under anesthesia with Avertin, and then small intestines, stomach contents and rectum feces were rapidly removed. According to the previous studies (Nishiyama *et al.* 2011a, b), IgA concentrations in stomach contents were represented as milk IgA level. The samples of stomach contents were pooled for all neonatal mice born to each mother at 14 dpp. The samples of small intestines were frozen in liquid nitrogen and stored at -80°C and the samples of stomach contents and rectum feces were stored at -20°C.

Blood samples from maternal or neonatal mice were left to stand at room temperature for 30 min and then centrifuged at 3000 rpm for 15 min or 10000 rpm for 5 min, respectively. The samples of serum were stored at -20°C.

IgA immunoassay and immunohistochemical analysis

IgA immunoassay of serum, stomach contents, small intestines and feces and immunohistochemical analysis of mammary glands, jejunum and ileum were determined as described in Chapter III. IgA concentrations were measured using the Mouse IgA ELISA Quantitation Kit (Bethyl Laboratories) and ELISA Starter Accessory Package (Bethyl Laboratories) according to the manufacturer's instruction.

The sections obtained from the immunohistochemical analysis were examined under an epifluorescence microscope (BX50, Olympus), and the resulting images were

analyzed by Image J software (National Institute of Health). The IgA-positive cells in the mammary glands were counted in eight randomised fields from each mouse and represented as IgA ASC/field of view (field = 1160 μm × 870 μm). Those in the jejunum and ileum were counted in lamina propria of villi in eight randomised villi from each mouse and represented as IgA ASC/unit area of lamina propria of villi (unit = 10000 μm^2).

Quantitative RT-PCR

The mRNA expressions of IgA C-region in the mammary gland and jejunum were examined by quantitative RT-PCR. Total RNA was extracted using RNeasy mini kit (Qiagen). cDNA was synthesised using 2 μg of total RNA with a SuperScript VILO cDNA Synthesis kit (Life technologies). Twenty-fold diluted cDNA (2 μl) was used as a template in a reaction with 0.2 μM of each primer and 1x SYBR$^{@}$ Select Master Mix (Life technologies) in a volume of 20 μL and each sample was analyzed in duplicate. Four serial dilutions were set up to determine Ct values and reaction efficiencies for all primer pairs.

Real-time PCR was performed using StepOnePlus Real-time PCR system (Life technologies) with the cycle parameters: 50°C for 2min and 95°C for 2min followed by forty cycles of PCR reaction at 95°C for 15s and 60°C for 1min. Melting curve analysis was performed after the amplification under the following condition: 95°C for 15s, 60°C for 1min, heat increment up to 95°C, and 95°C for15s. The primers used for IgA C-region were the same as those in Chapter III. The amount of IgA C-region mRNA was normalized to GAPDH by the $\Delta\Delta C_T$ method. The primers for GAPDH were as follows: forward: 5'-TGTGT CCGTC GTGGA TCTGA-3', reverse: 5'-CCTGC

TTCAC CACCT TCTTGA-3'. The data analysis was performed with the StepOne Software V 2.3.

Statistical analysis

Data from bodyweight and feed intake of maternal mice and bodyweight of neonatal mice during prepartum or postpartum periods were analyzed by least squares ANOVA using the general linear models procedure of SAS (1997). The model was as follows:

$$Y_{ijk} = \mu + T_i + M_{(i)j} + D_k + TD_{ik} + e_{ijk}$$

where μ is the overall mean, T_i is the effect of treatment, $M_{(i)j}$ is the random variable of a mice nested in treatment, D_k is the effect of day, TD_{ik} is the interactions, and e_{ijk} is the residuals. The general linear model procedure of Statistical Analysis Systems (SAS 1997) was used to analyse the effects of treatment on some variables in maternal mice and neonatal mice. Significance was declared at $P<0.05$.

RESULTS

IgA concentrations in serum and tissues

Bodyweight gains and feed intake of maternal mice during prepartum and postpartum periods were not affected by the treatment (Figure 5-1). Bodyweight gains of neonatal mice were not affected by the treatment (data not shown). IgA concentrations in serum, stomach contents, small intestines and feces in neonatal nice at 14 dpp were not affected by the treatment (Table 5-1), and. serum IgA concentrations in maternal mice at 14 dpp were not affected by the treatment (data not shown).

IgA antibody-secreting cells and expression of mRNA in tissues

In maternal mice, the numbers of IgA ASC in the jejunum of the α-tocopherol group at 14 dpp were significantly higher ($P<0.05$) than those of the control group, but the numbers of IgA ASC in the ileum and mammary glands were not affected by the treatment (Table 5-2). The mRNA expressions of IgA C-region in the jejunum, ileum and mammary glands of maternal mice were not affected by the treatment.

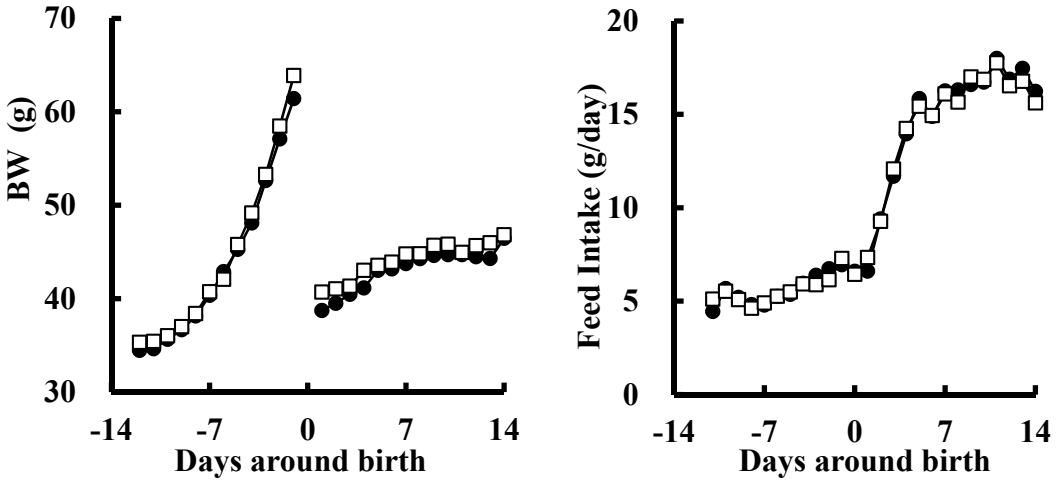
DISCUSSION

IgA antibodies in guts are specific for antigens of the intestinal microflora and act to limit penetration of commensal intestinal bacteria through the neonatal intestinal epithelium (Harris *et al*. 2006). Supplementation of vitamin E has been shown to enhance immune responses in animals, and the dietary vitamin E requirement for optimum T- and B-lymphocyte responses to mitogens was greater than 50 mg/kg in weanling rats (Bendich *et al.* 1986). Supplemental vitamin E in amounts of 60-180 mg/kg increased humoral immune response in mice, although normal diets contained 50-60 mg/kg vitamin E (Tengerdy *et al*. 1973). Also, high dietary intakes in amounts of 100-2500 mg/kg diet of vitamin E modified the functions of splenic lymphocytes and alveolar macrophages in rats (Moriguchi *et al*. 1990). In the present study, supplemental α-tocopherol at 120 mg/kg diet increased IgA ASC in the jejunum of lactating mice, although the numbers of IgA ASC in the ileum and mammary glands were not affected by the treatment. Because normal feed contained 91mg/kg vitamin E, dietary intake at

210 mg/kg vitamin E may be useful for enhancing IgA ASC in the jejunum of lactating mice.

β-carotene and vitamin E are often found in vegetables, and high-quality vegetables or silages contain large amounts of β-carotene and vitamin E. Supplemental β-carotene at 50mg/kg in the diet in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the mammary gland and ileum and of milk IgA during lactation, and that the effects may be mainly due to the RA-mediated immune response (Nishiyama *et al*. 2011a). However, supplemental α-tocopherol at 120 mg/kg diet had no effect on IgA concentrations in stomach contents of neonatal mice in the present study. Thus, compared with β-carotene, supplemental α-tocopherol may have little effect on IgA transfer from maternal milk to neonatal mice.

a) Mother



b) Neonate

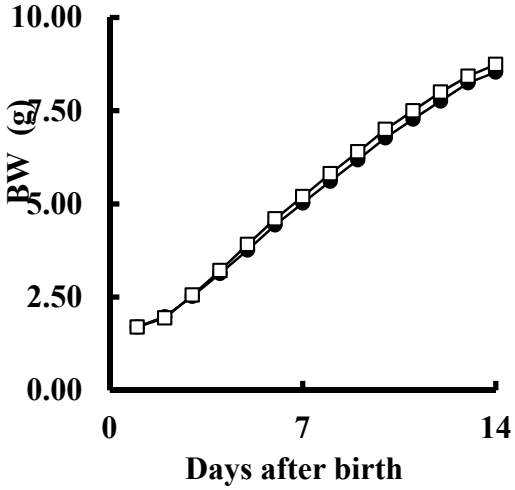


Figure 5-1. Bodyweight (BW) and feed intake of maternal mice and bodyweight of neonatal mice around parturition of the control (□) and α-tocopherol (●) groups during prepartum and 14 days postpartum.

Table 5-1. IgA concentrations in serum of maternal mice and serum, stomach contents, small intestine and feces of neonatal mice in the control and α-tocopherol groups at 14 days postpartum (Mean ± SE).

	Control	α-tocopherol	*P*
IgA, μg/g			
Mother			
Serum	1690 ± 401	1969 ± 357	NS
Neonate			
Serum	1.9 ± 0.3	1.5 ± 0.2	NS
Stomach contents	251 ± 30	248 ± 24	NS
Intestines	447 ± 77	625 ± 130	NS
Feces	4882 ± 667	6769 ± 1234	NS

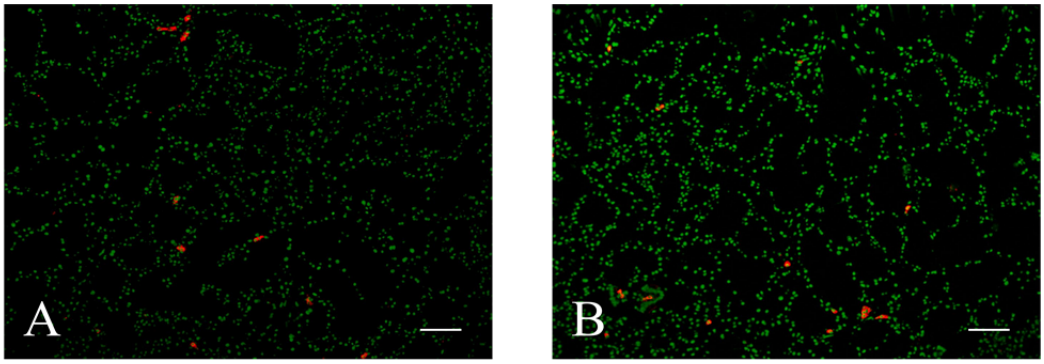


Figure 5-2. Immunofluorescent staining of IgA ASC in the mammary glands of lactating mice in the control (A) and α-tocopherol (B) groups at 14 days postpartum. IgA staining is shown in red, and nuclei are shown in green. Bar = 100 μm

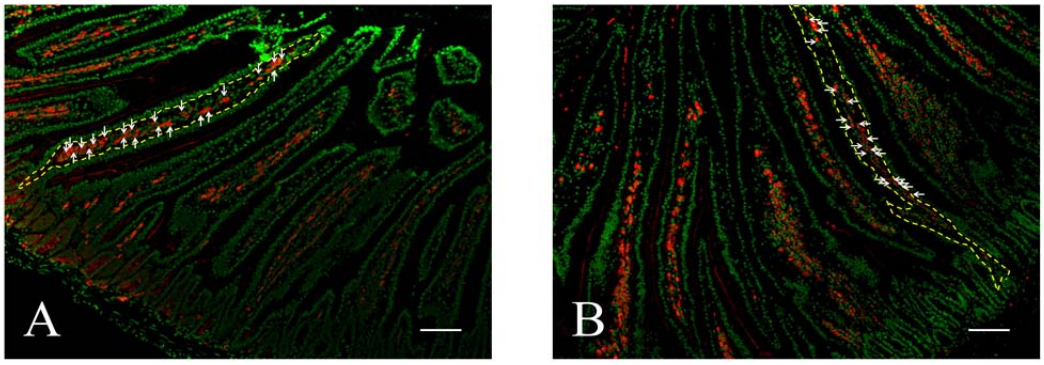


Figure 5-3. Immunofluorescent staining of IgA ASC in the jejunum of lactating mice in the control (A) and α-tocopherol (B) groups at 14 days postpartum. IgA staining is shown in red, and nuclei are shown in green. The enclosed area by yellow dashed line represents the lamina propria of villi, and arrows represent IgA ASC. Bar = 100 μm

Table 5-2. Numbers of IgA ASC and the ratios of IgA C-region mRNA to GAPDH mRNA in the mammary glands, jejunum and ileum of maternal mice in the control and α-tocopherol groups at 14 days postpartum (Mean ± SD).

	Control	α-tocopherol	*P*
IgA ASC			
Mammary glands	7.0 ± 0.5	6.7 ± 0.7	NS
Jejunum	10.5 ± 0.5	12.6 ± 0.4	*
Ileum	10.8 ± 0.3	11.1 ± 0.3	NS
IgA mRNA			
Mammary glands	1.69 ± 0.32	1.67 ± 0.55	NS
Jejunum	0.47 ± 0.15	0.30 ± 0.10	NS
Ileum	0.66 ± 0.13	0.86 ± 0.30	NS

* $P<0.05$

Chapter VI

General Discussion

IgA serves as the first line of defense in protecting the intestinal mucosa from enteric toxins and pathogenic microorganisms. In mammalian animals, IgA in colostrum or milk can protect the newborn neonates from gastrointestinal infections (Mantis *et al*. 2011). However, the exact mechanism of IgA production in the intestine and mammary glands of mammals during lactation is still unclear. In this study, I evaluated the relationships between fat-soluble vitamins and colostral IgG or IgA production in Japanese Black multiparous cows and clarified the effects of supplemental coumestrol, β-cryptoxanthin and α-tocopherol on IgA production in the intestine and mammary glands of lactating mice.

In Chapter II, data from 19 Japanese Black multiparous cows were collected to clarify the relationships among IgG, IgA, β-carotene, vitamin A and α-tocopherol contents in colostrums of cows in order to evaluate the role of fat-soluble vitamins on colostral IgG and IgA production. Mean colostral IgG was 141 mg/mL, ranging from 65 to 208 mg/mL, whereas mean colostral IgA was 8.7 mg/mL, ranging from 1.0 to 34.6 mg/mL. Colostral IgG increased with aging in multiparous cows. There were positive correlations between colostral IgG and colostral vitamin A or colostral α-tocopherol in cows, and the higher adjusted R^2 was obtained in the prediction model of colostral IgG from age and colostral vitamin A. Colostral vitamin A was positively correlated with colostral β-carotene or colostral α-tocopherol in cows, but there were no relationships between colostral IgA and colostral IgG or colostral fat-soluble vitamins. These results

indicate that fat-soluble vitamin contents in colostrum of cows may change in similar patterns and high colostral vitamin A is related with high colostral IgG.

Provitamin A (β-carotene) and vitamin E are mainly found in pasture and in grass and legume silages, but synthetic vitamins are often supplemented in diets of cows (Johansson *et al.* 2014; Kume & Toharmat 2001). Passive immune protection of the newborn gastrointestinal tract is dependent on an active process of IgA ASC accumulation in the mammary glands of the mother (Morteau *et al.* 2008; Nishiyama *et al.* 2011b). In cows, a large portion of colostral IgA is locally synthesized in the mammary glands, whereas IgG in the colostrum and milk is derived from blood circulation by a selective accumulation (Guidry *et al*. 1980; Larson & Gillespie 1957). Both IgA and IgG plasma cells are derived from IgM positive B cell, but the class switches of Ig are regulated by different signaling pathways. Because the positive relationships between colostral IgG and colostral vitamin A or colostral α-tocopherol were found in Japanese Black multiparous cows, feeding high-quality silage in pregnant cows is useful to increase not only colostral fat-soluble vitamins but also colostral IgG. On the other hand, although high amount of IgA were found in colostrum, colostral IgA was not related with fat-soluble vitamins.

In Chapter III, to clarify the effects of coumestrol administration in maternal mice during pregnancy and lactation on IgA ASC in the intestine and mammary glands in lactating mice, maternal mice were administered at 200 μg/kg body weight/day of coumestrol from 6.5 to 16.5 days postcoitus and 1 to 13 dpp. Coumestrol administration increased the numbers of IgA ASC and mRNA expressions of IgA C-region and VCAM-1 in the mammary glands of maternal mice at 14 dpp, but coumestrol administration had no effect on the numbers of IgA ASC in the ileum. Coumestrol

administration increased serum IgA concentration in maternal mice at 14 dpp, but IgA concentrations in serum, stomach contents, intestines and feces of neonatal mice were not affected by treatment. These results imply that coumestrol administration in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the mammary glands during lactation owing to the activated mRNA expressions of IgA C-region and VCAM-1 in the mammary glands.

In Chapter IV, to clarify the effects of supplemental β-cryptoxanthin in maternal mice during pregnancy and lactation on IgA ASC in the intestine and mammary glands of lactating mice, maternal mice were fed rodent feed or 50 mg/kg β-cryptoxanthin-supplemented rodent feed from 6.5 days postcoitus to 7 or 14 dpp. Supplemental β-cryptoxanthin increased the numbers of IgA ASC and the mRNA expressions of IgA C-region, CCL25 and CCL28 in the jejunum at 14dpp. Supplemental β-cryptoxanthin had no effect on the numbers of IgA ASC in the ileum and mammary glands, although supplemental β-cryptoxanthin increased the mRNA expression of IgA C-region in the ileum and mammary glands at 14dpp. Supplemental β-cryptoxanthin had no effect on IgA concentrations in serum, stomach contents, intestines and feces of neonatal mice. These results imply that supplementation of β-cryptoxanthin in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the jejunum during late lactation.

In Chapter V, to clarify the effects of supplemental α-tocopherol in maternal mice during pregnancy and lactation on IgA ASC in the intestine and mammary glands of lactating mice, maternal mice were fed rodent feed or 120 mg/kg α-tocopherol-supplemented rodent feed from 6.5 days postcoitus to 14 dpp. Supplemental α-tocopherol increased the numbers of IgA ASC in the jejunum at 14dpp,

but supplemental α-tocopherol had no effect on the numbers of IgA ASC in the ileum and mammary glands. Supplemental α-tocopherol had no effect on IgA concentrations in serum, stomach contents, intestines and feces of neonatal mice. These results imply that supplemental α-tocopherol in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the jejunum.

Hormones may enhance the milk IgA and pIgR levels in the mammary glands during lactation (Halsey *et al*. 1982; Rincheval-Arnold *et al*. 2002), and some effects of estrogen were related to protection against oxidative injury of blood vessels (Mori *et al*. 2004). Estradiol plays an important role in regulating uterine IgA, and the increase in the IgA level that occurs spontaneously during the estrus cycle is probably due to the action of estradiol in the uterus (Wira & Sandoe 1980). In the present study, coumestrol may act on ERs and increase IgA ASC in the mammary glands by activating the homing ligand VCAM-1 in the mammary tissue.

Several effects of carotenoids are thought to be mediated by their metabolism to vitamin A and subsequent mediation of RAR and RXR response pathways (Rühl 2007). Supplemental β-carotene at 50mg/kg in the diet in maternal mice during pregnancy and lactation increased the numbers of IgA ASC and mRNA expression of IgA C-region in the ileum during lactation, but in the mammary glands, β-carotene supplementation only increased the numbers of IgA ASC (Nishiyama *et al*. 2011a). Supplemental β-carotene increased the mRNA expressions of CCL25 and IgA ASC in the jejunum of weanling mice after 14 and 21 days of treatment, and these effects may be mainly due to the RA-mediated immune response (Nishida *et al*. 2014). In the present study, β-cryptoxanthin may increase the homing of IgA ASC in the jejunum by activating the expression of chemokine ligand CCL25 in the intestine.

Antioxidants prevent cell damage against free radicals and are critical for maintaining health, and dietary antioxidants have been related to modulate the host susceptibility or resistance to infectious pathogens (Puertollano *et al*. 2011). Supplementation of astaxanthin-enriched yeast in the diet increased the numbers of IgA ASC in the jejunum and ileum and IgA concentrations in the ileum of weanling mice (Nagayama *et al*. 2014). On the other hand, the supplementation of vitamin E at 250 mg/kg in the diet increased IgA antibodies in the intestine and serum in chickens (Muir *et al*. 2002). In the present study, supplemental α-tocopherol may be useful to increase the IgA ASC in the jejunum in lactating mice.

In conclusion, the present study clarified that vitamin A and α-tocopherol in colostrum were correlated with colostral IgG in Japanese Black multiparous cows, but colostral IgA was not related with fat-soluble vitamins. Coumestrol administration in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the mammary glands during lactation owing to the activated mRNA expressions of IgA C-region and VCAM-1 in the mammary glands. On the other hand, supplementation of β-cryptoxanthin in maternal mice during pregnancy and lactation is effective to increase the numbers of IgA ASC in the jejunum during late lactation owing to the active mRNA expression of CCL25 in the intestine.

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