Supplemental β-carotene increases IgA secreting cells in mammary gland and IgA transfer from milk to neonatal mice

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Abbreviations: ASC, antibody secreting cells; GALT, gut-associated lymphoid tissue; RA, retinoic acid.

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Mortality of neonates continues to be major problems in human and animals, and IgA provides protection against microbial antigens at mucosal surfaces. Although β-carotene supplementation has been expected to enhance retinoic acid-mediated immune response in neonates, the exact mechanism of β-carotene for enhancing IgA production is still unclear. We investigated the effect of supplemental β-carotene for maternal mice during pregnancy and lactation on IgA antibody secreting cells (ASC) in mammary gland and guts of maternal mice and IgA transfer from milk to neonatal mice. Pregnant mice were fed untreated or 50 mg/kg of β-carotene supplemented diets from 6.5 days postcoitus to 14 days postpartum. Supplemental β-carotene increased the numbers of IgA ASC in mammary gland (P<0.05) and ileum (P<0.001) and also mRNA expression of IgA C-region in ileum (P<0.05) of maternal mice at 14 days postpartum, but few numbers of IgA ASC were detected in mammary gland at 17.5 days postcoitus. IgA concentration in stomach contents, which represented as milk IgA, was significantly higher (P<0.01) in neonatal mice born from β-carotene supplemented mother at 7 and 14 dpp, and IgA concentration in serum, stomach contents and faeces increased (P<0.001) drastically with age. These results suggest that β-carotene supplementation for maternal mice during pregnancy and lactation is useful for enhancing IgA transfer from maternal milk to neonates due to the increase of IgA ASC in mammary gland and ileum during lactation.

Mortality and morbidity of neonates continue to be major problems in human and animals, and their most common disease is diarrhoea. Supplemental vitamin A and β-carotene enhance the immune system in neonates, and β-carotene has the highest pro-vitamin A activity[1-5]. Vitamin A deficiency is associated with an increased risk of death from common childhood infections, and supplementation of vitamin A decreases diarrhoea and mortality in malnourished children[6, 7]. Additionally, β-carotene deficient calves had a higher incidence of diarrhoea and mortality in the first week of life[8, 9]. Foods containing pro-vitamin A carotenoids are the primary source of vitamin A, and β-carotene is very rich in some vegetables or fruits[10,11]. Because animals are not able to synthesize retinoids *de novo*[11], supplementation of β-carotene enriched foods may be effective to enhance the immune system in human and animals.

Passive immunity is critical to the survival and health of neonates, and colostrum or milk is a source of nutrients and immune components for the neonates. IgA is the most abundant Ig isotype in mucosal secretions and provides protection against microbial antigens at mucosal surfaces[6,12-14]. IgA antibodies produced from IgA antibody secreting cells (ASC) in mammary glands are secreted mainly as dimers after incorporation of the J chain and association with a transmembrane epithelial glycoprotein known as polymeric-Ig receptor[12,15]. IgA antibodies in milk are specific for antigens of the intestinal microflora and acts to limit penetration of commensal intestinal bacteria through the neonatal intestinal epithelium[16, 17]. Therefore, passive immune protection of the newborn gastrointestinal tract is dependent on an active process of IgA ASC accumulation in the lactating mammary gland of the mother[18].

The gut-associated lymphoid tissue (GALT) is the largest immunologic organ in the body. Peyer's patches are the main site for the generation of IgA^+ B cells, and plasmablasts differentiated by IgA^+ B cell home preferentially to the gut lamina propria through the thoracic duct and blood[6,12-14]. Recent studies[19,20] showed that vitamin A metabolite, all-trans retinoic acid (RA), play important roles in gut immunity and RA is necessary for the imprinting of

gut-homing specificity on T cells and the induction of gut-homing receptors on B cells and IgA ASC. Several effects of carotenoids are thought to be mediated by their metabolism to vitamin A and subsequent mediation of RA receptor and retinoid X receptor-response pathways[21]. Mice and rats efficiently convert β-carotene to vitamin A but absorb carotenoids intact only when they are provided in the diet at supraphysiologic levels[10]. Thus, mice may be more appropriate animal models for investigating supplemental β-carotene on IgA production in mammary glands. However, the exact mechanism of β-carotene for enhancing IgA transfer from mother to neonates is still unclear, although β-carotene supplementation has been expected to enhance RA-mediated immune response in neonatal mice.

We investigated the effect of supplemental β-carotene for maternal mice during pregnancy and lactation on the number of IgA ASC and mRNA expression of IgA C-region in mammary gland and guts of maternal mice and IgA transfer from maternal milk to neonatal mice. The present study demonstrated that β-carotene supplementation for maternal mice during pregnancy and lactation is effective to increase the number of IgA ASC in mammary gland and ileum and milk IgA during lactation, and their effects may be mainly due to the RA-mediated immune response. Additionally, most IgA in neonatal mice may be derived from milk IgA and β-carotene supplementation enhances IgA transfer from maternal milk to neonatal mice.

Materials and Methods

Animals and diets

Pregnant ICR mice (n=28) were purchased from Clea Japan (Tokyo, Japan). They were housed in polycarbonate cages and maintained in an air-conditioned room (24±2℃) under controlled lighting conditions (light: dark cycle, 14:10 h). All mice were allowed free access to water and treated in accordance with ”Regulation on Animal Experimentation at Kyoto University” (Animal Research Committee. Kyoto University, revised 2007).

Maternal mice were fed untreated (control group) or 50 mg/kg of β-carotene supplemented diets (β-carotene group) from 6.5 days postcoitus (dpc) to 14 days postpartum (dpp). The diets contained the vitamin mix, but no detectable amounts of β-carotene were presented in the vitamin mix. Seven maternal mice from each group were dissected at 17.5 dpc (maternal mice during pregnancy) and 14 dpp (maternal mice during lactation). At birth, average litter size of mice born from control and β-carotene group was 13.9 and 15.0, respectively. All neonatal mice were alive by 7 dpp, and subsets of neonatal mice in each group, except 5 female and 5 male neonatal mice born from each mother, were dissected at 7 dpp. Then, 5 female and 5 male neonatal mice born from each mother were dissected at 14 dpp.

Sample collection

Body weights of maternal mice before birth and food intake of maternal mice throughout the experiment were measured at 10.00 o'clock every day. Body weights of neonatal mice were measured at 10.00 o'clock every day. Blood samples from maternal mice at 17.5 dpc and 14 dpp were obtained by cardiac puncture under anaesthesia by Avertin (2,2,2-tribromoethanol, Sigma-Aldrich Chemical, MO, USA), and then mammary gland, jejunum and ileum were removed and 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℃ for semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Blood samples from neonatal mice at 7 and 14 dpp were obtained by incising their hearts and collecting with haematocrit tubes under anaesthesia by Avertin, and then small intestine, stomach contents and rectum faeces were rapidly removed. At 7 dpp, samples of blood, small intestine, stomach contents and rectum faeces of neonatal mice born from each mother were pooled, and samples were separately pooled by female or male neonatal mice born from each mother at 14 dpp. The samples of small intestine were frozen in dry ice-cooled isopentane for immunohistochemical

analysis or frozen in liquid nitrogen and stored at -80℃ for IgA analysis and semi-quantitative RT-PCR. The samples of stomach contents and rectum faeces were stored at -20℃ until IgA analysis.

IgA Immunoassay

Blood samples in maternal or neonatal mice were put stable at room temperature (RT) for 1 hour or 30 min and then centrifuged at 3,000 rpm for 15 min or 10,000 rpm for 5 min, respectively. Serum was fractionated for IgA analysis. Stomach contents and rectum faeces were thawed, strongly vortexed in cold PBS containing protease inhibitor (complete Mini, Roche, Basel, Switzerland) and centrifuged at 15,000 rpm for 20 min at 4℃. Small intestine was homogenized using Sample Grinding Kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instruction and centrifuged at 15,000 rpm for 10 min at 4℃. Each supernatant was fractionated for IgA analysis.

IgA concentration was measured using the Mouse IgA ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, USA) and ELISA Starter Accessory Package (Bethyl Laboratories) according to the manufacturer's instruction. Plates obtained from the procedures were read at 450 and 620 nm with a Microplate Reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA).

Immunohistochemical Analysis

Fresh frozen sections (6μm 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 RT. After washing in phosphate-buffered saline (PBS, pH 7.4), the sections were incubated with 1% BSA and 5% normal donkey serum in PBS containing 0.02% Tween20 (blocking solution) for 20 min,

successively with rabbit anti-mouse IgA (Open Biosystems, AL, USA; 1:400 in the blocking solution) for 2 h at RT. After washing in PBS containing 0.02% Tween20 (0.02% PBST), the sections were incubated with Alexa fluor 555 donkey anti-rabbit IgG (invitrogen, Calisbad, CA, USA; 1:500 in the blocking solution) for 90 min at RT. The sections were washed in 0.02% PBST, mounted with glycerol (Wako Pure Chemicals) and then examined under a confocal laser scanning microscope (FV300, Olympus, Tokyo, Japan). The resulting images were analyzed by ImageJ software (National Institute of Health, Bethesda, MD, USA).

The IgA-positive cells in mammary gland were counted in randomized eight fields from each mouse and represented as IgA ASC/field of view. Those in jejunum and ileum were counted in lamina propria of villi in randomized 5-8 villi from each mouse and represented as IgA ASC/unit area of lamina propria of villi (unit=10,000μm^2).

Semi-quantitative RT-PCR

The mRNA expression of IgA C-region in the tissue was examined by Semi-quantitative RT-PCR. Total RNA was extracted, using RNeasy mini kit (Qiagen, Maryland, CA, USA). Complementary DNA (cDNA) was synthesized with oligo (dT) primer using SuperScriptⅢ First-Strand Synthesis System for RT-PCR (invitrogen) from 4 μg RNA of each samples. The PCR was performed using Platinum PCR SuperMix kit (invitrogen). The primer pairs for IgA C-region were as follows: F: 5'-TGCACAGTTACCCATCCTGA-3', R: 5'- GCACCAGCACTTCTTTAGGG -3'. PCR cycles were as follows: after 95℃ for 7 min to denature DNA, PCR performed for 35 cycles at 95℃ for 1 min, 53℃ for 1 min, 72℃ for 1 min, then for 30 cycle of most tissues of maternal mice or 35 cycle of mammary gland of pregnant mice and intestine of neonatal mice at 72℃ for 5 min. 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 semiquantified using ImageJ software. The relative abundance of specific

mRNA was normalized by abundance of GAPDH mRNA.

Statistics

Data were expressed as mean values with their standard error. Data from body weight and feed intake were analyzed by least squares ANOVA using the general linear models procedure of SAS[22]. 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 the effect of treatment, $M_{(i)j}$ the random variable of a mice nested in treatment, D_k the effect of sampling day, TD_{ik} the interactions, and e_{ijk} the residuals. The general linear models procedure of SAS[22] was used to analyze the effects of treatment on some variables in maternal mice or neonatal mice at 7 dpp, the effects of treatment, sex and their interaction on some variables in neonatal mice at 14 dpp, and the effect of age on some variables in neonatal mice. Significance was declared at $P<0.05$.

Results

Body weight and food intake

Body weight gains and food intake of maternal mice dissected at 14 dpp as well as those of maternal mice dissected at 17.5 dpc (data not shown) were similar between groups (Fig. 1). In maternal mice, body weights increased ($P<0.001$) during pregnancy and food intake increased ($P<0.001$) rapidly after birth. Body weight gains of neonatal mice were almost similar between groups, but body weights at 7 and 14 dpp were significantly lower ($P<0.01$) in neonatal mice born from β-carotene supplemented mother.

IgA concentration in serum and tissues

Serum IgA concentrations of maternal mice at 17.5 dpc and 14 dpp were not affected by treatment (Table 1). Compared to IgA concentration in serum of neonatal mice at 7 dpp, serum IgA concentration of maternal mice was about 1,000 times higher. IgA concentrations in stomach contents of neonatal mice born from β-carotene supplemented mother were significantly higher (P<0.01) than that of control mice at 7 and 14 dpp. Although no significant differences were obtained in serum, small intestine and faeces of neonatal mice, their IgA concentrations were slightly higher in mice born from β-carotene supplemented mother at day 14 (Table 1). Compared to IgA concentration of neonatal mice at 7 dpp, IgA concentrations in serum (P<0.001), stomach contents (P<0.001), small intestine (P<0.01) and faeces (P<0.001) increased drastically at 14 dpp, but their IgA concentrations were not affected by sex.

IgA ASC in tissues

The numbers of IgA ASC in jejunum and ileum of maternal mice at 17.5 dpc were not affected by treatment (Table 2). The numbers of IgA ASC in mammary gland (P<0.05) and ileum (P<0.001) of maternal mice fed β-carotene were significantly higher than that of control mice at 14dpp, but there was no significant difference in jejunum. Few numbers (2<) of IgA ASC were detected in mammary gland of maternal mice at 17.5 dpc and jejunum and ileum of neonatal mice at 14 dpp in each group (data not shown).

Expression of mRNA in tissues

The mRNA expression of IgA C-region in ileum was significantly higher (P<0.05) in maternal mice fed β-carotene at 14dpp, but there were no differences in mammary gland and jejunum (Fig. 4). At 17.5 dpc, there were no significant differences in mRNA expression of IgA C-region in mammary gland, jejunum and ileum of maternal mice between treatments. The mRNA expression

of IgA C-region in jejunum significantly decreased (P<0.01) in neonatal mice born from β-carotene supplemented mice at 14dpp, but that was not affected by sex.

Discussion

The importance for adequate consumption of high quality colostrum or milk on acquisition of optimal nutrition and passive immunity is widely recognized in neonates[1,23]. The present study indicated that the nutritional status in maternal and neonatal mice was thought to be normal, because the pattern of food intake and growth rate of maternal or neonatal mice agrees with the previous reports[16,24] and low body weights in mice born from β-carotene supplemented mother may be due to the more pups at birth. Supplemental β-carotene for maternal mice during pregnancy and lactation increased the number of maternal IgA ASC in mammary gland during lactation, although very few numbers of IgA ASC were detected during pregnancy. Additionally, higher IgA concentration in stomach contents, which represented as IgA level in milk[25], was observed in neonatal mice born from β-carotene supplemented mothers.

The majority of Ig in murine milk belongs to IgA and milk IgA provides protection against microbial antigens in neonates[15]. GALT-dendritic cells rely on RA for inducing IgA class switching and RA is essential for the imprinting of gut-homing receptors on T and B cells[19,20]. Supplementation of vitamin A and carotenoids affect the immune-cell function during ontogenesis, and higher values of total serum IgG were found in β-carotene enriched (300mg/kg) neonatal mice on day 7[26]. Vitamin A-depleted mice show the impaired IgA secretion and protection at mucosal tissues[20]. Additionally, 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 faecal IgA of neonatal mice[18]. Compared to the increase of milk IgA, β-carotene supplementation had no clear effect on faecal IgA in this study. However, because faecal IgA at 14 dpp was 1.6 times higher in mice born from β-carotene supplemental

mother, 50 mg/kg of β-carotene may be slightly useful to enhance faecal IgA via the increase of milk IgA. Thus, our data imply that β-carotene supplementation for maternal mice during pregnancy and lactation is effective to increase the number of IgA ASC in mammary gland and milk IgA during lactation, and their effects may be mainly due to the RA-mediated immune response because mice efficiently converted β-carotene to vitamin A[10].

The pathways leading to milk IgA production is complex, but most IgA in lactating mice is derived from the serum by day 4 of lactation and IgA synthesis by mammary cells becomes most important during late lactation[27]. The mammary gland develops new vasculature and is colonized by lymphocytes during pregnancy, and it is colonized primarily by IgA-containing B cells during lactation[28]. As a result, milk IgA transfers the ability from mother to neonatal mice to provide exclusion of luminal bacteria, and this process is not critically dependent on antibody specificity[16]. Compared to day 7 in neonatal mice, the rapid increase of IgA concentrations was observed not only in stomach contents but also in serum, small intestine and faeces at day 14 in the present study. On the other hand, the deceased mRNA expression of IgA C-region in jejunum in neonatal mice born from β-carotene supplemented mother at day 14 may be partly due to the delay in neonatal mucosal immune induction. However, the intestinal secretions of IgA in mice at weaning could hardly be found, and then the amounts of IgA rose drastically and reached a maximum concentration at 10 weeks of age[29]. Because very few numbers of IgA ASC were detected in jejunum and ileum of neonatal mice at day 14, most IgA in neonatal mice may be derived from milk IgA and β-carotene supplementation enhance IgA transfer from maternal milk to neonatal mice.

IgA plasma cells in the mammary gland in mice are derived from the lymphoid cells in GALT by homing to the mammary gland[17]. Homing of ASC to the intestinal mucosa requires the expression of integrin α4β7[6,14]. Retinoic acid is important to induce α4β7 and CCR9 on activated T cells and blocking RA-receptors decreases the induction of gut-homing receptors[19]. We

demonstrated that β-carotene supplementation increased mRNA expression of IgA C-region and the number of IgA ASC in the ileum during lactation, but in the mammary gland, β-carotene supplementation increased only the number of IgA ASC. In murine small intestine, mature isolated lymphoid follicles are inductive sites for the immune response and the nodular lymphoid structures are observed in the distal small intestine [30]. The number of IgA ASC has been greatly reduced in the small bowl of vitamin A deficient mice, but retinoids are not absolutely required for IgA production in tissues other than the small intestine[6,20]. Additionally, maternal IgA ASC, primed in the gut and respiratory tract, home to the mammary gland during late pregnancy and lactation[17,29], and IgA ASC from the mammary gland express CCR10 and migrate CCR28 during lactation[31]. These results suggest that β-carotene supplementation have a predominant effect on IgA production in the ileum and the increased IgA ASC in the ileum is homing to the mammary gland, resulting in the increased IgA ASC in the mammary gland and milk IgA. However, further study is needed to clarify the exact mechanism of β-carotene for homing from the ileum to the mammary gland.

In conclusion, the present study suggested that the supplementation of β-carotene for maternal mice during pregnancy and lactation is useful for enhancing IgA transfer from maternal milk to neonates due to the increase of IgA ASC in mammary gland and ileum of maternal mice during lactation.

Acknowledgments

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353

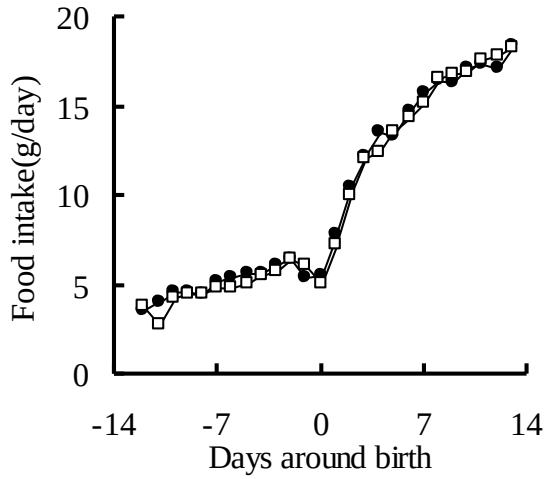
354 **Fig. 1** Least squares means of body weight and feed intake of maternal mice in control (●)

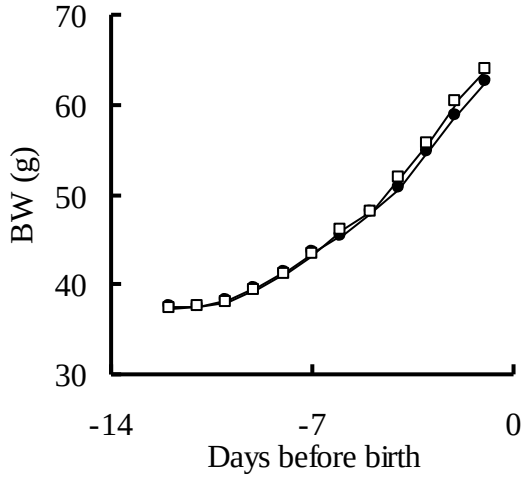
355 and β-carotene group (○), and body weight of their neonatal mice.

356

357

a) Mother





b) Neonate

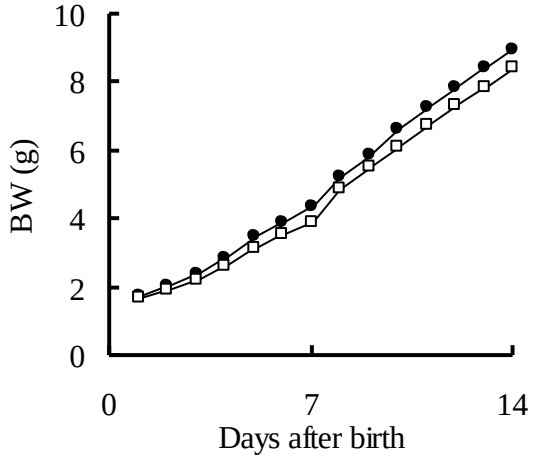


Fig. 1

Table 1. IgA concentration (μg/g) in serum of maternal mice at 17.5 days postcoitus (dpc) and 14 days postpartum (dpp) and serum, stomach contents, intestine and faeces of neonatal mice at 7 and 14 dpp in control and β-carotene group.

		Control		β-carotene	
	Days	Mean	SE	Mean	SE
Mother					
Serum	17.5dpc	630	120	690	121
	14dpp	479	79	715	102
Neonate					
Serum	7dpp	0.38	0.03	0.42	0.03
	14dpp	2.35	0.21	2.77	0.28
Stomach	7dpp	12.2[a]	2.3	26.6[b]	3.5
	14dpp	38.8[a]	4.5	73.3[b]	9.1
Intestine	7dpp	28.0	9.7	34.0	10.6
	14dpp	84.1	18.8	129.6	22.4
Faeces	7dpp	71.2	25.8	71.4	19.1
	14dpp	356	69	567	110

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0.01$).

Table 2. The numbers of IgA ASC and mRNA expression of IgA C-region in mammary gland, jejunum and ileum of maternal mice at 17.5 days postcoitus (dpc) and 14 days postpartum (dpp) and the mRNA expression of IgA C-region jejunum and ileum of neonatal mice at 14 dpp in control and β-carotene group.

		Control		β-carotene	
	Days	Mean	SE	Mean	SE
The numbers of IgA ASC					
Mammary gland	14dpp	8.8[a]	0.7	12.0[b]	1.0
Jejunum	17.5dpc	7.5	0.4	8.0	0.3
	14dpp	7.7	0.6	8.8	1.0
Ileum	17.5dpc	7.6	0.5	8.0	0.2
	14dpp	7.0[e]	0.4	9.6[f]	0.5
IgA mRNA/GAPDH					
Mother					
Mammary gland	17.5dpc	0.86	0.07	0.94	0.12
	14dpp	0.63	0.04	0.71	0.03
Jejunum	17.5dpc	1.01	0.09	1.04	0.05
	14dpp	1.38	0.17	1.68	0.28
Ileum	17.5dpc	0.78	0.10	0.90	0.04
	14dpp	0.82[a]	0.08	1.29[b]	0.17
Neonate					
Jejunum	14dpp	1.29[c]	0.09	0.95[d]	0.08
Ileum	14dpp	1.53	0.21	1.73	0.29

[a,b]Mean values within a row with unlike superscript letters were significantly different ($P<0.05$).
[c,d]Mean values within a row with unlike superscript letters were significantly different ($P<0.01$).
[e,f]Mean values within a row with unlike superscript letters were significantly different ($P<0.001$).
The numbers of IgA ASC in mammary gland were counted in randomized eight fields from each mouse, and values in jejunum and ileum were counted in lamina propria of villi in randomized 5-8 villi from each mouse. The mRNA expression represents relative IgA C-region mRNA expression normalized by abundance of GAPDH mRNA.