JARQ 59 (1), 61-69 (2025) https://www.jircas.go.jp

Investigation of the Effects of Antigen Administration to the Mother during Pregnancy and Lactation on the Immune Responses of Offspring Using the Antigen Hypersensitive Mouse Model

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Abstract

Food antigens ingested by the mother can sensitize the offspring via the mother, though how the child's immune system responds to them is poorly understood. This is because mothers also can produce antibodies to ingested food antigens that can be transferred to the child, and it is difficult to distinguish between such maternal antibodies and antibodies produced by the child's immune system in response to maternal antigens. In this study, we examined the effects of antigens ingested by pregnant and lactating mothers on the immune responses of their offspring using DO11.10, introducing a T cell receptor gene that recognizes the ovalbumin peptide. Since DO11.10, unlike wild-type mice, is strongly sensitized even by oral administration of ovalbumin alone, we have successfully induced allergic predisposition in DO11.10 by oral administration of ovalbumin in the past. Hence, we used the combination of a wild-type mother and DO11.10 heterozygous offspring to investigate the effects of mother-derived antigens on the offspring's immune system while minimizing the mother's immune response. We found that antigen-specific antibodies in the sera of offspring of mothers which ingested antigens during pregnancy and lactation and the class of antibodies in the offspring depended on the antigen ingestion by the mother. Furthermore, offspring from mothers which received continuous oral antigen ingestion during lactation had higher levels of serum antigen-specific IgE induced by sensitization after growth than those of mothers which did not ingest the antigen. In contrast, when the offspring of allergic DO11.10 mothers were given the same antigen orally after maturation, antibody titers did not differ significantly from those of offspring of non-allergic DO11.10 mothers. These results suggest that the antigen ingestion by the mother during pregnancy and lactation affects the immune response of their offspring.

Discipline: Food
Additional key words: breastfeeding, fetus, IgE, immunological memory, oral antigen

Introduction

In recent years, the number of pediatric food allergy patients has continued to increase, and some of these patients will likely develop other allergic diseases such as atopy or asthma; therefore, effective countermeasures are urgently needed. Although numerous studies have examined the underlying mechanism of immediate allergic symptoms, the mechanism by which children develop allergic diathesis remains unclear. The rapid increase in patients in recent years cannot be explained solely by genetic traits, and environmental factors seem critical. Environmental pollution, social stress, and microbial infection in early childhood are the likely causes of infant food allergy. Still, in addition to these factors, exposure to maternal food antigens in utero and through breast milk have also been postulated as causes. Peanuts ingested by the mother during pregnancy reportedly can sensitize the offspring and induce IgE production (Sicherer et al. 2010). Another study showed that the immune system of the offspring of pregnant women infected with pathogens becomes activated (Guadalupe et al. 2009). Breast milk reportedly contains sufficient antigens to induce allergy in infants (Kilshaw

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Received 25 December 2023; accepted 26 April 2024.

& Cant 1984). Other human studies have shown that some allergic children have elevated IgE titers (Han et al. 2009) or worsening of symptoms (Cavagni et al. 1988) due to exposure to food allergens through breast milk. However, some animal studies have shown that breastfeeding induces antigen-specific oral immune tolerance in offspring (Bednar-Tantscher et al. 2001, El-Merhibi et al. 2012, Polte & Hansen 2008, Tooley et al. 2009, Verhasselt et al. 2008).

Human breast milk contains immune-suppressive cytokines such as transforming growth factor–β, which can induce antigen-specific immune tolerance in breastfed offspring (Halken 2004). Recent evidence clearly indicates that the intestinal microbiota exerts a strong influence on the immune system. Oligosaccharides in breast milk have been shown to stimulate the growth of intestinal bacteria that benefit health by modulating immunity (Selvamani et al. 2023). These data indicate that breastfeeding could contribute to reducing the risk of developing allergies in offspring. However, other studies have reported that sensitization in utero or through breast milk is not associated with allergy development in offspring (Pali-Scholl et al. 2009, Venter et al. 2009).

These findings indicate that the impact of food antigens ingested by the mother on the child's immune response through the uterus and breast milk remains highly controversial. In humans, controlling the conditions involved in allergy development is difficult. Even in animal studies, it can be difficult to evaluate only the child's immune response during the fetal and infant stages resulting from exposure to maternal food antigens because the mother herself becomes sensitized to the antigens she has ingested and produces antibodies, which are then transferred to her child.

In the present study, we analyzed the effects of maternal antigens on the offspring's immune response using an allergic mouse model we previously established (Goto et al. 2009). It is nearly impossible to induce antigen-specific antibodies in wild-type mice through oral administration of small amounts of antigen without oral adjuvants such as cholera toxin (Snider et al. 1994, Czerkinsky et al. 1989). However, we have demonstrated that DO11.10 can induce ovalbumin (OVA)-specific antibodies through oral administration of only a small amount of OVA (Goto et al. 2009). DO11.10 is a BALB/c mouse background that introduces a VDJ-reconstituted T cell receptor (TCR) gene that recognizes the OVA peptide (Murphy et al. 1990). The method for oral administration of OVA used in this study does not stimulate a sufficient immune response to induce detectable OVA-specific antibodies in BALB/c mice. However, it can induce specific antibodies in DO11.10 mice. Therefore, we crossed a DO11.10 homozygous father with a BALB/c mother to obtain DO11.10 heterozygous offspring. Each T cell is adjusted to express the reconstituted TCR from only one of the alleles by an allelic exclusion mechanism (Malissen et al. 1992, Outters et al. 2015) so that each T cell corresponds to a single antigen. Thus, DO11.10 mice express only the introduced TCR, even in the heterozygote. Thus, it was hypothesized that oral administration of OVA to the BALB/c mother could sensitize only the DO11.10 offspring through the mother, minimizing the production of antibodies by the mothers. The model was expected to enable us to exclude the effects of the mother's immune response when analyzing that of the offspring.

Materials and methods

1. Materials

(1) Animals

Female BALB/c mice were purchased from Charles River Japan. DO11.10 mice carrying the chicken egg OVA-specific T cell receptor gene were purchased from Jackson Laboratory (Boston, MA, USA) and maintained in our specific pathogen-free animal facilities. Animals were fed a standard diet of rodent chow and water *ad libitum*. All animal studies were reviewed and approved by the Animal Care and Use Committee of the National Agriculture and Food Research Organization (NARO), Japan (approved number H24-41).

(2) Chemicals and Reagents

OVA (Fraction V grade) was purchased from Sigma (St. Louis, MO, USA). OVA for oral administration was purchased from Wako Pure Chemical Industries (Osaka, Japan). Blocking buffer and horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulins for enzyme-linked immunosorbent assay (ELISA) use were purchased from Bethyl (Montgomery, TX, USA). TMB Microwell ELISA-Peroxidase substrate was purchased from Surmodics (Eden Prairie, MN, USA). The chemiluminescent substrate for the HRP enzyme, FEMTOGLOWPlus, was purchased from Michigan Diagnostic (LLC, Royal Oak, MI, USA). All other chemicals were of the highest purity available from commercial sources.

2. Oral antigen administration

For intermittent oral antigen administration, the mice were given a 2% (w/w) aqueous OVA solution as drinking water for three days, followed by pure water for four days. Then, they were given a 2% (w/w) aqueous OVA solution for three days *ad libitum* (Dosing Method 1). For continuous oral antigen administration, the mice

were given a 2% (w/w) aqueous OVA solution as drinking water for six days, followed by pure water for four days *ad libitum* (Dosing Method 2) (Fig. 1A). After switching the type of drinking water, the rearing cages were changed to minimize the remaining OVA.

3. Antigen administration to pregnant mothers

To obtain more than three offspring, mother mice that had previously given birth were used. BALB/c female mice were mated with DO11.10 homozygotes male mice. The mothers were subjected to Dose 1 starting 10 days after mating. The non-sensitized group was provided pure water. DO11.10 heterozygous offspring born 21 to 24 days after mating were weaned at three weeks, and sera collected at four weeks. Dose 1 was then subjected to offspring that had reached 10 to 14 weeks of age, and sera was collected on day 11 after antigen administration. These collected sera were stored at −30°C until use (Fig. 1B).

4. Antigen administration to nursing mothers

To obtain more than three offspring, mother mice that had previously given birth were used. BALB/c female mice were mated with DO11.10 homozygous male mice. Immediately after giving birth, the mothers were subjected to oral OVA administration (Dosing Method 1 or 2), and the non-sensitized group was given pure water. DO11.10 heterozygous offspring were weaned at three weeks, and sera were collected at four weeks. Dosing Method 1 was then subjected to offspring that had reached 10 to 14 weeks, and sera were collected on day 11 after antigen administration. These collected sera were stored at −30°C until use (Fig. 1C).

The experimental conditions were designed based on physiological and behavioral considerations to minimize the possibility of the offspring ingesting OVA directly from drinking water or via the mother's feces.

5. Administration of antigen to offspring from allergic mothers

To obtain more than three offspring, DO11.10 homozygous mother mice that had previously given birth were used. Mother mice were subjected to Dose 1, which induces OVA-specific IgE (Goto et al. 2009); these were mothers with allergic predisposition, i.e., allergic mothers. The mother mice that received Dose 1 were

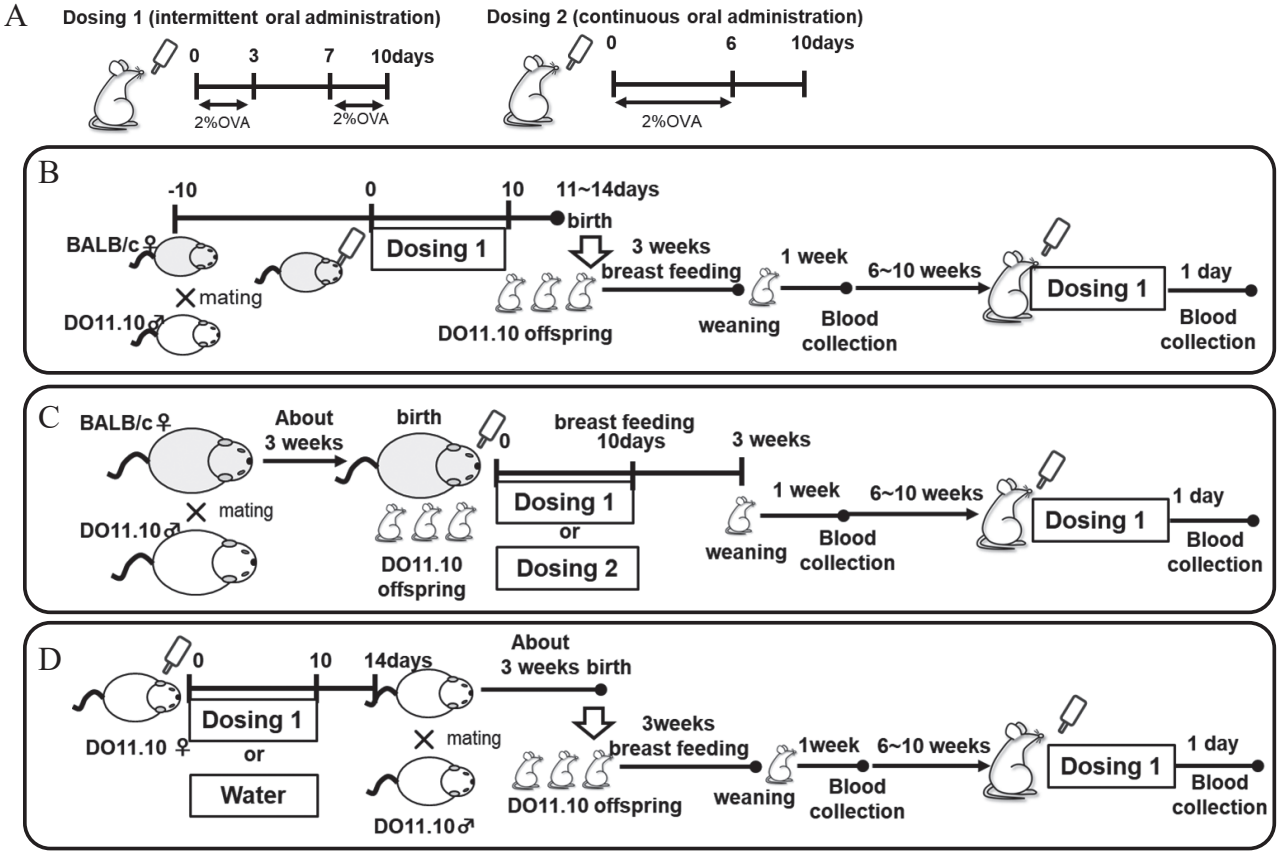

Fig. 1. Experimental designs

A. Dosing method, B. Administration of OVA during pregnancy, C. Administration of OVA during breast feeding, D. Investigation of offspring from allergic mothers

"sensitized mothers," and those that received pure water were deemed as "control." Four days after the treatment, the mother mice were mated with DO11.10 homozygous male mice. The resulting offspring were weaned at three weeks of age and were subjected to Dose 1 at 10 to 14 weeks. Sera were collected on day 11 after antigen administration and stored at −30°C until use (Fig. 1D).

6. Measurement of serum antibodies

OVA-specific antibodies in sera were measured using a sandwich ELISA. Maxisorp plates (NUNC, Boston, MA, USA) were coated with OVA in phosphate-buffered saline ([PBS] 100 μg/ml) at 4°C overnight. Serum samples diluted with a blocking buffer were added to the plate wells and incubated at 4°C overnight. The plates were washed with wash buffer (PBS containing 0.05% Tween 20), followed by the addition of an HRP-labeled anti-mouse immunoglobulin and incubation for 1 h at room temperature. A TMB substrate was added to each plate, and the absorbance was measured (450 nm) after stopping the reaction.

For OVA-specific IgE measurement, C96 white Maxisorp plates (NUNC) were used. Serum samples were diluted with solution 1 of the Can Get Signal kit (TOYOBO Biochemicals, Osaka, Japan). An HRP-labeled anti-mouse IgE diluted with Can Get Signal solution 1 was added, and the plates were incubated for 1 h at room temperature. FEMTOGLOWPlus was added to each well, and the reaction was measured.

Standard curves were prepared by stepwise dilution of sera obtained from OVA-orally administered (Dosing Method 1) DO11.10 mice that were not used in this work. The titer of each antibody in the standard serum was set as IgG1: 16,000 Units, IgG2a: 42,000 Units, and IgE: 1,200 Units, respectively. Serum samples from non-sensitized DO11.10 mice not used in this work were used to evaluate non-specific responses in the ELISA system.

7. Statistical analysis

Statistical comparisons were performed using Student's two-tailed *t*-test. A P-value < 0.05 indicates a significant difference compared to the control. If the measured value was smaller than the detection limit, it was expressed as not detected (N.D.), and no significance difference test was performed.

Results

1. Evaluating the effects of oral antigen administration to pregnant mothers on the immune response of their offspring

To investigate the effect of oral antigen administration to pregnant mothers on the immune system of their offspring, antigen-specific antibodies of the offspring after weaning were measured (Fig. 1B). OVA-specific IgG1, IgG2A, and IgE were detected in offspring from mothers who received Dosing Method 1. In the case of offspring from mothers which did not ingest OVA, OVA-specific IgG1 and IgG2A titers were not detected. IgE titers were significantly low, comparable to the sera of non-sensitized mice (Fig. 2).

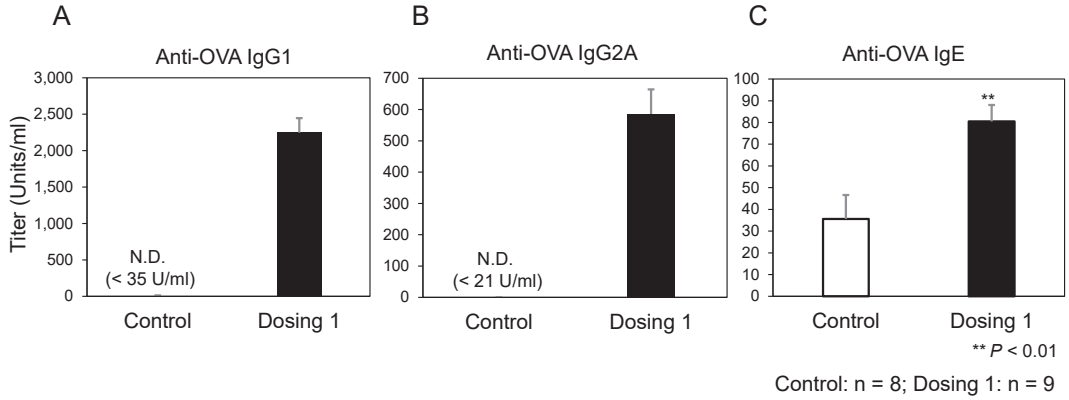

Fig. 2. Effect of intermittent antigen administration to pregnant mothers on the antigen-specific antibodies in the sera of offspring after weaning (4 weeks old)

Titers of anti-OVA antibodies (IgG1, IgG2A, and IgE) in serum from offspring evaluated by ELISA. Data represent results for the offspring of mothers who were not treated (Control: open columns) and offspring of mothers which were OVA-administered intermittently during pregnancy (Dosing Method 1: closed columns) in two independent experiments. Data shown in A, B, and C are expressed as mean ± standard error (SE).

**Significantly different from the control (offspring from non-exposed mothers) (P < 0.01). If the value of the control was not detected, a significance difference test was not performed.

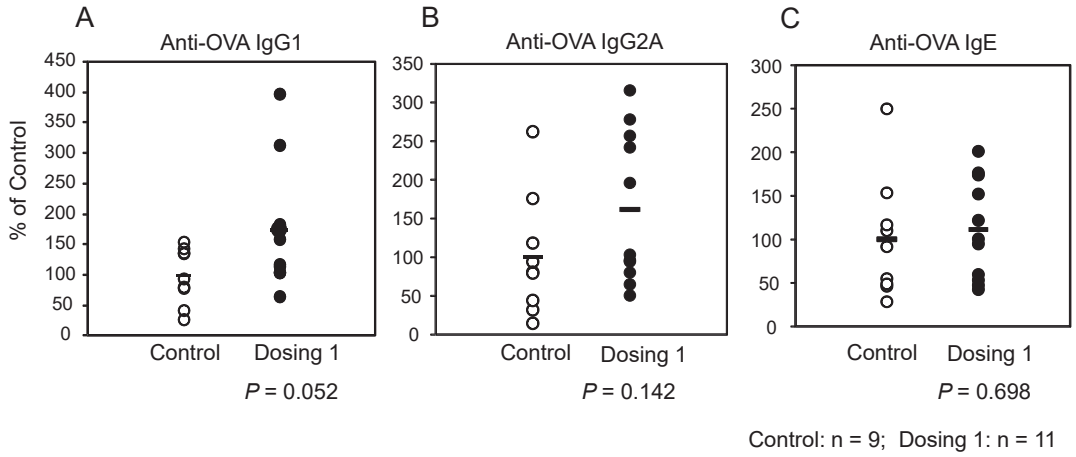

Fig. 3. Effect of antigen administration to pregnant mothers (Dosing Method 1) on the antigen-specific antibody production in postgrowth offspring sensitized orally
Titers of anti-OVA antibodies (IgG1, IgG2A, and IgE) in the sera of postgrowth offspring immunized with oral OVA. Horizontal lines represent means. Antibody titers are expressed as 100% of the mean of the antibody titer of the control group (offspring from non-OVA administered mothers).

To determine the effect of oral OVA administration to pregnant mothers on the immune responses of the offspring after growth, these offspring at 10 to 14 weeks old were subjected to Dosing Method 1, and OVA-specific antibodies in sera were measured (Fig. 1B). While we confirmed that OVA-specific antibodies could not be detected in the sera collected just before oral administration of OVA in all offspring (data not shown), even after growth, offspring born to mothers which received OVA orally during pregnancy tended to have higher serum IgG1 titers than those born to mothers which did not receive OVA (Fig. 3).

2. Evaluating the effects of oral antigen administration to nursing mothers on the immune responses of their offspring

To investigate the effect of oral antigen administration to nursing mothers on the immune system of offspring (Fig. 1C), antigen-specific antibodies in the sera of offspring after weaning were measured. OVA-specific IgG1 was detected in the offspring of mothers subjected to Dosing Method 1 (Fig. 4). IgG2a titers were not detected, and IgE titers were significantly low, comparable to non-sensitized mice sera (data not shown). In contrast, IgG1 and IgE were detected in the offspring of mothers subjected to Dosing Method 2 (Fig. 5). To determine the effect of oral OVA administration to nursing mothers on the immune responses of their offspring after growth, these offspring at 10 to 14 weeks were subjected to Dosing Method 1, and OVA-specific antibodies in their sera were measured. We confirmed that OVA-specific antibodies could not be

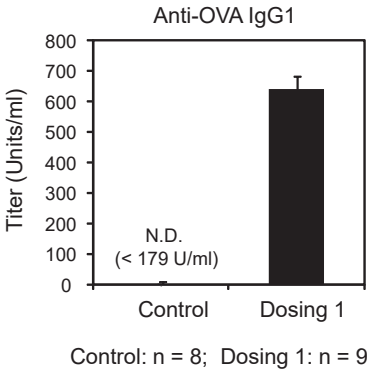

Fig. 4. Effect of antigen administration to nursing mothers (Dosing Method 1) on the antigen-specific antibodies in offspring after weaning (4 weeks old)
Titer of anti-OVA IgG1 in serum from offspring, as determined by ELISA. Data represent offspring of not-treated mothers (control: open columns) and OVA-administered mothers (Dosing Method 1: closed columns) in four independent experiments. Data are expressed as mean ± SE.
Because the value of the control was not detected, significance difference test was not performed.

detected in the serum collected just before oral administration of OVA in all offspring (data not shown). Although the antibody titers of offspring born to mothers subjected to Dosing Method 1 did not differ from those of offspring of mothers which did not receive antigens (Fig. 6), the offspring of mothers subjected to Dosing Method 2 had significantly higher antigen-specific IgE titers than those of offspring of mothers which did not receive antigens (Fig. 7).

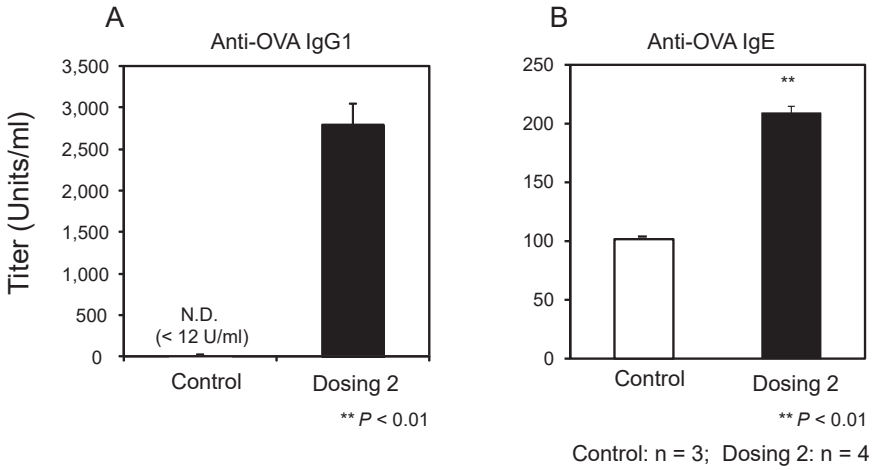

Fig. 5. Effect of antigen administration to nursing mothers (Dosing Method 2) on the antigen-specific antibodies in offspring after weaning
Titers of anti-OVA IgG1 and IgE in serum from offspring, as determined by ELISA. Data represent offspring of not-treated mothers (control: open columns) and OVA-administered mothers (Dosing Method 2: closed columns) in two independent experiments. Data in A and B are expressed as mean ± SE.
**Significantly different from the control (offspring from not OVA administered mothers) ($P < 0.01$). Because the value of the control was not detected, a significance difference test was not performed.

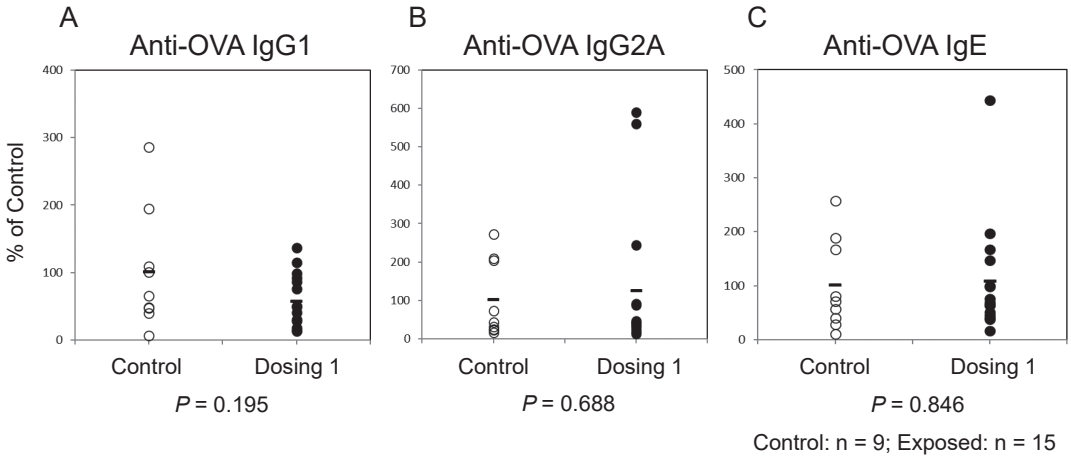

Fig. 6. Effect of antigen administration to nursing mothers (Dosing Method 1) on the antigen-specific antibody production in postgrowth offspring sensitized orally
Titers of anti-OVA antibodies (IgG1, IgG2A, and IgE) in serum of grown offspring from mothers administered OVA (Dosing Method 1) or not (Control) immunized with oral OVA intermittently. Horizontal lines represent means. Antibody titers are expressed as 100% of the mean of the antibody titer of the control group (offspring from non-OVA administered mothers).

3. Immune responsiveness of offspring born to allergic mothers

DO11.10 homozygous female mice were subjected to Dosing Method 1 to induce OVA-specific IgE (Goto et al. 2009) and then mated with DO11.10 homozygous male mice. The resulting offspring were subjected to Dosing Method 1 at 10 to 14 weeks (Fig. 1D). The antibody production of offspring born to allergic mothers did not differ significantly from those born to non-sensitized mothers (Fig. 8).

Discussion

It has been reported that ingested antigens can sensitize fetuses and alter their immune response (Sicherer et al. 2010, Guadalupe et al. 2009). We also

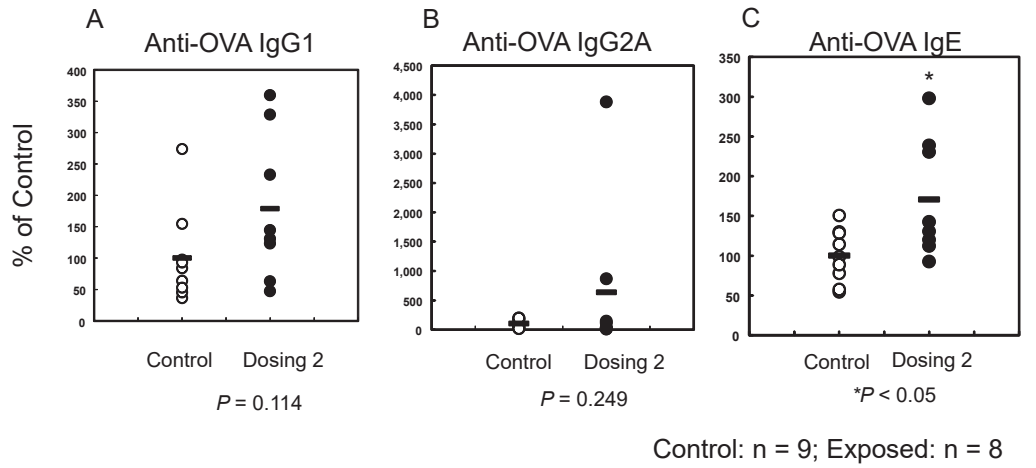

Fig. 7. Effect of antigen administration to nursing mothers (Dosing Method 2) on the antigen-specific antibody production in postgrowth offspring sensitized orally

Titers of anti-OVA antibodies (IgG1, IgG2A, and IgE) in serum of grown offspring from mothers administered OVA (Dosing Method 2) or not (Control) immunized with oral OVA intermittently. Horizontal lines represent means. Antibody titers are expressed as 100% of the mean of the antibody titer of the control group.

*Significantly different from the control (offspring from not administered mothers) ($P < 0.05$).

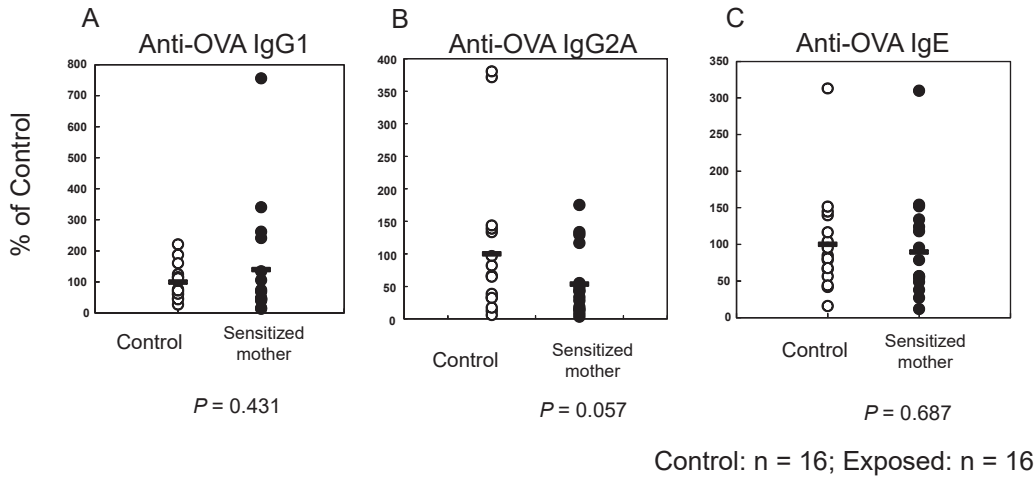

Fig. 8. Effect of maternal allergic state on the development of antigen-specific antibodies in postgrowth offspring with sensitized orally

Titers of anti-OVA antibodies (IgG1, IgG2A, and IgE) in serum of grown offspring from DO11.10 mothers which sensitized (Dosing Method 1) or not (Control) before mating. Horizontal lines represent means. Antibody titers are expressed as 100% of the mean of the antibody titer of the control group (offspring from non-sensitized mothers).

attempted to estimate the effect of maternal dietary antigen exposure on the immune response of fetuses. OVA-specific antibodies were detected in the sera of offspring from mothers which received intermittent oral administration of OVA during pregnancy (Fig. 2). These offspring were born 21 to 25 days after mating. The antigen was administered to mothers 10 to 20 days after mating (Fig. 1B). The thymus gland of mice is formed at approximately 11 days post-embryo in mice, and T cells are produced by 14 days post-embryo (Tenno et al. 2018). This suggests that T cells that could be sensitized were present in the fetus at the time of antigen administration to the mothers. Therefore, we sensitized grown offspring and evaluated the production of specific antibodies since re-sensitized individuals tend to have greater antibody production than those initially sensitized. As a result,

titers of OVA-specific antibodies induced by sensitization in adulthood tended to be higher in offspring from mothers which were orally administered OVA during pregnancy than in those from mothers which were not administered (Fig. 3). This suggests that offspring of mothers which ingested antigens during pregnancy may become sensitized to the antigens via the placenta.

On the contrary, it has been proposed that antigen-specific T cells are transferred from the fetus to the mother by microchimerism (Comitre-Mariano et al. 2022). According to such proposals, OVA-specific T cells from DO11.10 fetuses may induce OVA-specific antibodies in the mother by orally administered OVA, which could flow into the fetus. Although the number of cells transferred via microchimerism has been reported to be minor, it is well known that the immune responses of pregnant mothers differ from the norm. As such, the effects may be worth considering.

It has been reported that breast milk brings sufficient antigens from the mother's diet to induce allergy in infants (Kilshaw & Cant 1984, Cavagni et al. 1988, Han et al. 2009). Therefore, we attempted to estimate the effect of maternal dietary antigen exposure on the immune response of suckling offspring. Antigen-specific antibodies were found in the sera of offspring of mothers which were administered OVA continuously during nursing (Fig. 5). Additionally, the grown offspring were sensitized orally and evaluated to have OVA-specific antibodies. Moreover, offspring from nursing mothers which were orally administrated OVA continuously had significantly higher IgE titers than those that were not administered upon sensitization after growth (Fig. 7). These results suggest strongly that offspring born to mothers which ingested OVA continuously during lactation were sensitized before weaning. Since these experiments were designed to minimize the opportunity for offspring to orally ingest OVA from the feces of mothers or OVA solution, the offspring were likely sensitized via breastfeeding to the OVA that the mother ingested. On the other hand, OVA-specific IgG1 was detected in the post-weaning sera of offspring born to mothers who received OVA intermittently during lactation (Fig. 4). However, OVA-specific antibodies induced by sensitization after growth did not differ significantly from those of offspring born to mothers which had not ingested OVA (Fig. 6). These results suggest that the intermittent administration of antigen to mothers during lactation may not have induced a sustained immune memory in the offspring or may have failed to sensitize them. In the latter case, since the OVA-specific antibodies in the sera of offspring after weaning were thought to be of maternal origin, the possibility could not be excluded that the mothers produced the antibodies due to physiological changes unique to pregnancy and lactation, such as microchimerism. Additionally, it should be noted that we cannot wholly exclude from this experiment offspring born to continuously administered mothers which were also sensitized through routes other than breast milk, such as skin. However, in any case, since the total amount of antigen that the offspring could ingest was estimated to be the same between Dosing Methods 1 and 2, it is clear that the immune responses of the offspring differed depending on the timing and interval of antigen administration to their mothers.

We examined changes in immune responses in offspring born to mothers which had previously developed allergic predispositions following oral sensitization. However, there was no significant difference between offspring born to non-allergic mothers and those born to allergic mothers in terms of the immune responses stimulated by Dosing Method 1 after growth (Fig. 8). This suggests that maternal constitutional changes resulting from allergy induction do not significantly affect the immune response of offspring after growth.

In DO11.10 mice, which are highly responsive to the antigen OVA, the sensitivity to the allergen of offspring was suggested to be influenced by the duration and timing of maternal exposure to antigens rather than by the mother's allergic constitution.

Acknowledgements

We thank Michiko Yamamoto and Yoshiko Toyama for providing technical assistance.

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