

Maternal Antigen Stimulation Downregulates via Mother's Milk the Specific Immune Responses in Young Mice

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Key Words

Allergy · Breast-feeding · Milk · Ovalbumin · Murine · Neonate

Abstract

Background: Different aspects of the vertical transfer of predisposition to allergy from mother to child have been investigated. An issue which is still largely open is the influence of breast-feeding by allergic mothers on the development of the allergic phenotype of the infant. In the current study we employed a murine ovalbumin (OVA) immunization model to investigate possible milk-borne influences of the mother's specific immune status on the primary immune response of the breast-fed pup.

Methods: Naïve and OVA-immunized female mice were mated simultaneously. Immediately after birth litters were exchanged between the immunized and the untreated mothers which allowed the evaluation of maternal influence exerted via milk only. At the age of 3 weeks the pups were injected with a single dose of OVA intraperitoneally and sacrificed 2 weeks later. Serum was obtained for the determination of total and OVA-specific IgE and IgG2a. In addition, lymphocyte proliferation was measured following OVA stimulation of the pups' splenocytes and lymph node cells. During the lactation period milk was collected from the mothers for evaluation of its total and OVA-specific immunoglobulin levels. **Re-**

sults: Breast-feeding of naïve pups by OVA-immunized mothers results in the suppression of the pups' specific IgE response as well as the downregulation of the OVA-induced proliferative response of the pups' lymph node cells and splenocytes. Additionally, splenocytes of naïve pups nursed by immune mothers show a decrease in IL-4 production compared to naïve pups nursed by naïve mothers, whereas the IFN- γ production is not altered.

Conclusion: We demonstrated the suppression of the pups' primary humoral and cellular response towards OVA by breast-feeding by mothers exposed to OVA shortly before pregnancy. It appears that such a transfer of the suppressive activity from mother to pups via milk directs the pups' immune response towards a Th1 and away from a Th2 type, thus avoiding the 'allergic' phenotype. Our study suggests that breast-feeding by mothers immune to an antigen may suppress the development of an allergic response to this antigen.

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Introduction

Genetical predisposition is to some extent responsible for the manifestation of allergic disorders, but environmental components starting already with intrauterine antigen exposure also play a role [1, 2]. The influence of various environmental factors on the occurrence of allergic

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diseases has been described, including the role of the season of birth [3], respiratory infections [4] and breast-feeding [5–7]. Breast-feeding was described to be prophylactic against atopic diseases of childhood and adolescence [8]. Additionally, prolonged breast-feeding resulted in a lower incidence of severe atopic diseases particularly with a family history of atopy [9].

Ruiz et al. [10] reported that significantly more children of allergic mothers developed allergic eczema than those of allergic fathers. Transmission of atopy at the chromosome locus 11q was shown to be detectable only through the maternal line [10, 11]. These studies suggest that an atopic mother can modify the development of the infant's immune response towards the allergic phenotype, possibly by factors experienced by the fetus in utero. Another possibility is the postnatal transfer of atopy-modifying factors via breast milk. The babies' immune system is influenced in utero by the presence of maternal antibodies, cytokines and cells, but the same components can also be transferred from mother to child in the process of breast-feeding.

In the first days of lactation human colostrum contains on average 3.3×10^6 cells/ml, comprising macrophages, polymorphonuclear leukocytes and lymphocytes [12, 13]. Several inflammatory cytokines, growth factors and also chemokines, which might be important for the recruitment of the leukocytes into milk [14, 15] were detected in milk in biologically relevant amounts [14–18].

Possible differences in the broad spectrum of the bioactive substances found in breast milk between normal and atopic mothers are not yet clearly defined. It is not known what bias towards potential development of atopic disease the breast-fed infants receive via the milk from their allergic mothers. The aim of our study was to reveal whether breast-feeding by mice immunized to favor a Th2 response has a detrimental, beneficial or no effect on the immune responses of the newborn. We used a mouse model of ovalbumin (OVA) immunization to study the transfer of putative immune response-modifying activity in milk.

Materials and Methods

Mice

Ten- to 12-week-old female and male BALB/c mice were purchased from Charles River (Sulzfeld, Germany) and kept at the Novartis Forschungsinstitut under specific pathogen-free conditions. The mice received conventional mouse feed (SSNIFF, Soest, Germany), which has been certified to be OVA-free, and water ad libitum.

Immunization with OVA

OVA at doses of 10 and 100 µg (grade V, Sigma, Vienna, Austria) adsorbed with aluminum hydroxide gel (alum, Serva, Heidelberg, Germany; 2 mg) was given to each mouse in 0.2 ml PBS for priming and boosting, respectively. Priming was done intraperitoneally while boosting was done subcutaneously 21 days after the first immunization. For an evaluation of antibody levels serum was collected on days 0, 12 (after priming) and 30 (after boost). The pups were immunized intraperitoneally with 5 µg OVA in alum per mouse at the age of 3 weeks. Two weeks later the pups' serum, splenocytes and lymph node cells were obtained for evaluation of immunoglobulin levels and for lymphocyte stimulation tests, respectively.

Timed Mating of the Animals and Litters

On day 21 after priming, OVA-immunized and untreated female mice were mated. In order to get timed pregnancies, the mice were mated over a period of 24 h, 4 female and 1 male mouse were kept in one cage and separated again after 1 day. The pregnant mice gave birth to one to ten pups 3 weeks later. The litters were exchanged between immunized and naïve mothers. The exchanged litters were readily accepted by their foster mothers. In addition to group A, the control group (the pups were born and nursed by untreated mothers), this procedure resulted in two additional experimental groups. In group B, there were pups born to immunized mothers and fed by control mothers. Consequently exposure to OVA and OVA-specific antibodies and cells could only take place in the uterus. In group C, the pups were born to nonimmunized mothers and fed by immunized mothers. Therefore, the modification of OVA-specific responses of the pups could take place only via the milk.

Milk Collection

During the nursing period milk was collected from every mother for the evaluation of immunoglobulin levels. For milking the mice were anesthetized with 25% Rompun and Ketavet 1:1, 50 µl per mouse (20–25 g) and 1 U oxytocin (Sigma, Vienna, Austria) per mouse was administered intraperitoneally. From each mouse 50–500 µl milk was collected using the mouse milking machine (fig. 1a, b) as described by Hoffmann et al. [19]. The milk was diluted 1:2 with RPMI and centrifuged at 200 g. The top fat layer was discarded and the whey was collected and stored at –20 °C for a maximal period of 3 weeks until evaluation of the antibody levels.

ELISA

Total and OVA-specific IgE and IgG2a levels were determined. Total serum levels were obtained using standard ELISA. Briefly, 96-well Nunc immunoplates (Polysorb, Nunc, Roskilde, Denmark) were coated with a rat IgG1κ Mab directed against ε heavy chain of mouse Ig (LO-ME-3, University Leuven, Belgium) for total IgE ELISA and goat anti-mouse IgG2a (Southern Biotechnology, Birmingham, Ala., USA) for total IgG2a ELISA. Monoclonal mouse IgE anti-DNP (SPE-7, BioMakor, Rehovot, Israel) was used as ELISA standard for IgE ELISA in the range of 6–800 µg/ml and mouse IgG2a (UPC-10, Sigma) was used as ELISA standard for IgG2a ELISA in the range of 0.002–1 µg/ml. Sheep anti-mouse IgE labeled with horseradish peroxidase (Binding Site, Birmingham, UK) was used as conjugate for the IgE ELISA and goat anti-mouse IgG2a labeled with horseradish peroxidase (Southern Biotechnology) was used as conjugate for IgG2a ELISA. The substrate consisted of 45 ml substrate buffer with 1 tablet of 15 mg *o*-phenyldiamine dihydrochloride (Sigma). The reaction was stopped after 30 min with 12.5% sulfuric acid.

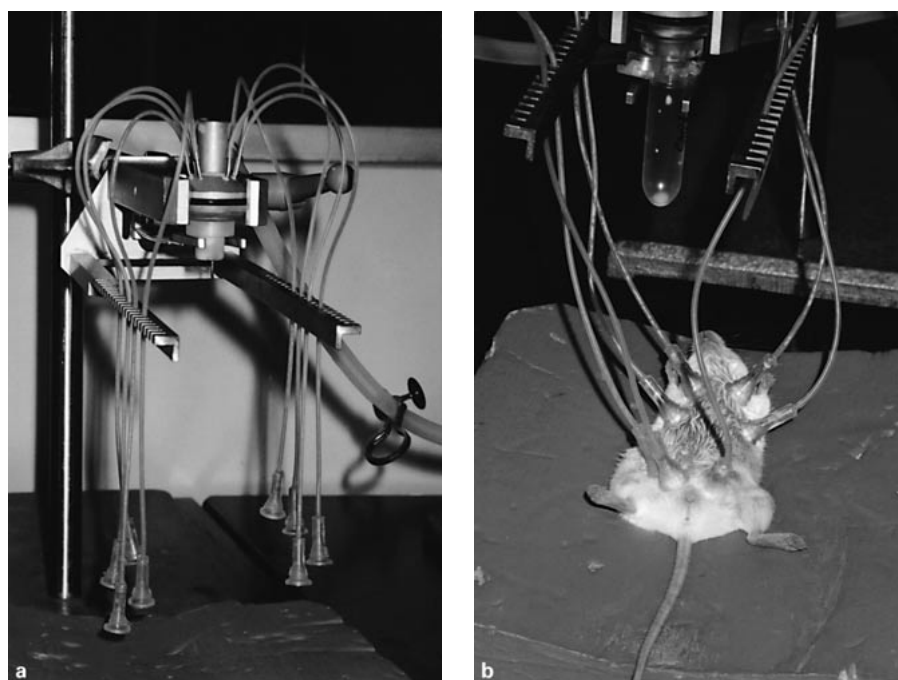


Fig. 1. **a** Apparatus used for milking mice. **b** Milking procedure.

Determination of OVA-specific IgE and IgG2a was performed using capture ELISA. Briefly, plates were coated as described for total IgE and IgG2a ELISA. The capture antibody used in the IgE ELISA was screened for cross-reactivity with other isotypes and was found to be 12% cross-reactive with purified IgG. Thus, the measured IgE levels in milk and serum cannot be due to cross-reactivity with other immunoglobulin isotypes. A serum pool of antigen-specific serum was used as standard. For OVA-specific IgE the serum pool was used in a range from 1:50 to 1:12,800 and for OVA-specific IgG2a the serum pool was used in a range from 1:100 to 1:10,240. Per definition, the serum dilution 1:100 equaled 100 capture units. Biotinylated chicken egg albumin was used as conjugate. Biotin was detected with streptavidin conjugated to alkaline phosphatase (Chemicon, Temecula, Ca., USA). A substrate kit from Bio-Rad (Hercules, Calif., USA) was used and the development was stopped with 2 N NaOH.

Lymphocyte Stimulation Test

Spleen and mesenteric lymph nodes were obtained from 5-week-old pups, 2 weeks after immunization. The organs were pooled from each individual litter, strained through a cell strainer (Falcon, Franklin Lakes, N.J., USA) and washed twice in PBS and penicillin/streptomycin (100 U/ml; 100 µg/ml). To separate the mononuclear cell population (MNC) from contaminating erythrocytes and granulocytes, the spleen single cell suspensions were layered over Lymphoprep (Nycomed, Oslo, Norway) and centrifuged at 800 *g* for 20 min at room temperature in a swing-out rotor without brakes. The MNC-containing interlayer was collected and washed twice in PBS. The cell count was determined and 10⁵ cells per well were put into culture in RPMI 1640 medium/10% fetal calf serum/100 U/ml penicillin/100 µg/ml streptomycin in 96-well microtiter plates (U-bottomed, Costar, N.Y., USA). Cells were stimulated with OVA (25 µg/ml) and

concanavalin A (Con A, 5 µg/ml) (Pharmacia, Uppsala, Sweden) in quadruplicates in a final volume of 200 µl. Cells were kept at 37°C and 5% CO₂. After 2 days (splenocytes) and 4 days (lymph node cells) of culture 1 µCi of methyl-³H-thymidine (Amersham, Little Chalfont, UK) was added to each well. 16 h later the cells were harvested (Harvester 96, Tomtec, Hamden, Conn., USA) and ³H-thymidine incorporation was determined in a scintillation counter (Wallac 1450, EG&G Wallac, Gaithersburg, Md., USA). Due to the differences in the proportions of lymphocytes, especially in spleen MNC preparations, the results were expressed as percent of response to Con A.

Cytokine Production by Cultured Splenocytes or Lymph Node Cells

Mononuclear single cell suspensions from spleen and lymph nodes (6 × 10⁵ cells per well) were incubated in RPMI 1640 medium/10% fetal calf serum/100 U/ml penicillin/100 µg/ml streptomycin in 96-well U-bottom microtiter plates and stimulated in quadruplicates with OVA (25 µg/ml) for 3 days. Supernatants were collected by pooling the quadruplicates and stored at -20°C for determination of cytokine levels.

IL-4 and IFN-γ ELISA

IL-4 and IFN-γ concentrations in culture supernatants were determined by ELISA according to the manufacturer's specifications (R&D Systems, Abingdon, UK).

Statistical Analysis

Student's *t* test with unequal variances was performed.

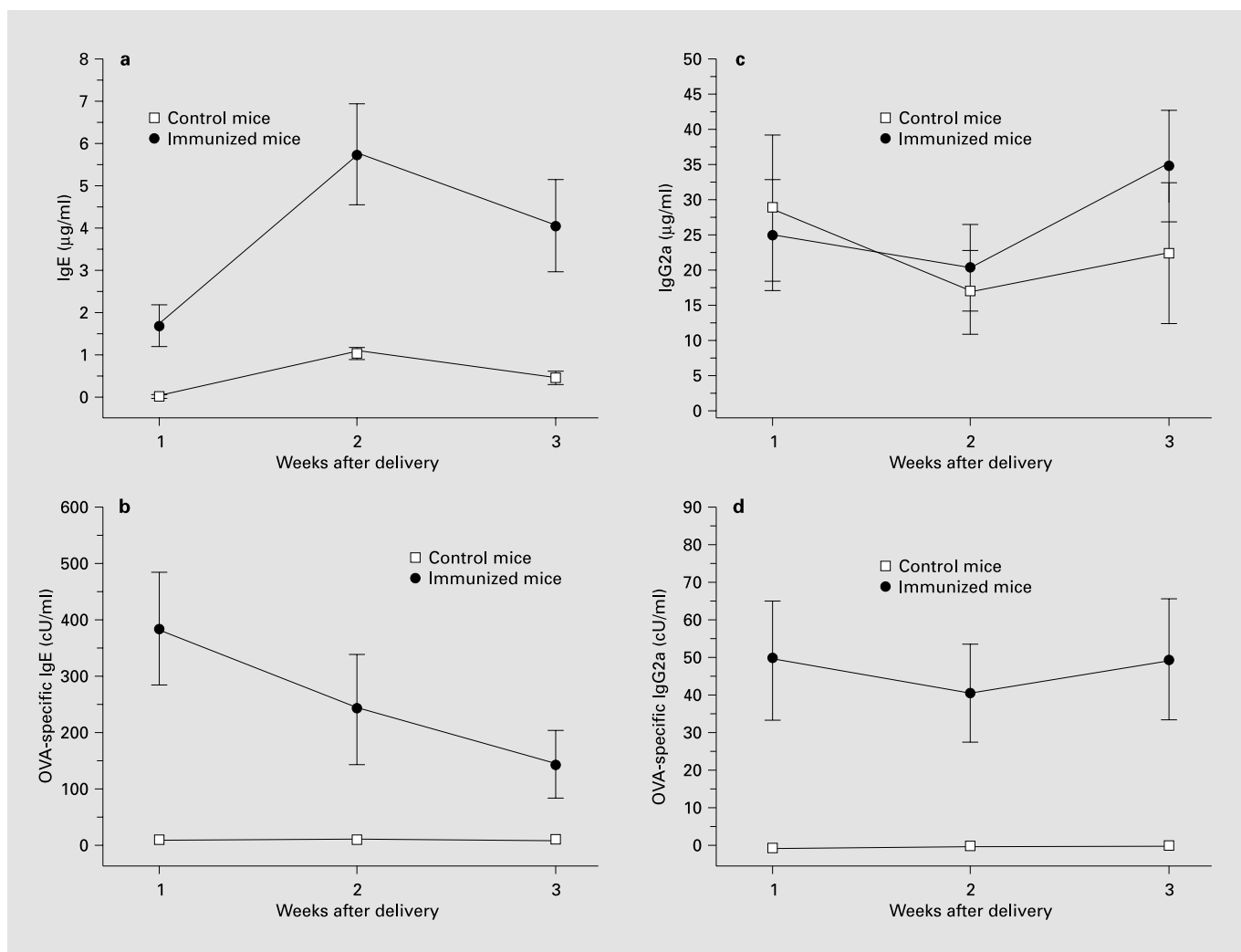


Fig. 2. Immunoglobulin levels in mouse whey: total IgE (**a**), OVA-specific IgE (**b**), total IgG2a (**c**) and OVA-specific IgG2a (**d**) (control mice: 8 animals, immunized mice: 11 animals; results are median values of immunoglobulins $\pm$ SEM). cU = Capture units.

Results

IgE and IgG2a in Mouse Milk

During the nursing period of approximately 20 days the mothers were milked 3 times. The total and OVA-specific IgE and IgG2a levels detected in mouse whey are shown in figure 2.

Total IgE levels in the milk of the control mice were low throughout the lactation period, whereas in immunized mice a maximum of nearly 6 μ g total IgE/ml could be observed in the 2nd week after delivery, which is also the time when lactation reaches its peak. No OVA-spe-

cific IgE could be detected in the whey of nonimmunized mice. In contrast, high levels of specific IgE were seen in the whey of immunized mothers in the 1st week postpartum, decreasing with time but still detectable at the end of lactation. Total IgG2a whey levels were similar in control mice and immunized mice, whereas OVA-specific IgG2a was detected only in the whey of immunized mice. The concentration of specific IgG2a remained unchanged throughout lactation. These results reveal that total as well as specific IgE and IgG2a are present in mouse milk after immunization and are transferred to the pups. Furthermore, a good correlation of total and OVA-specific

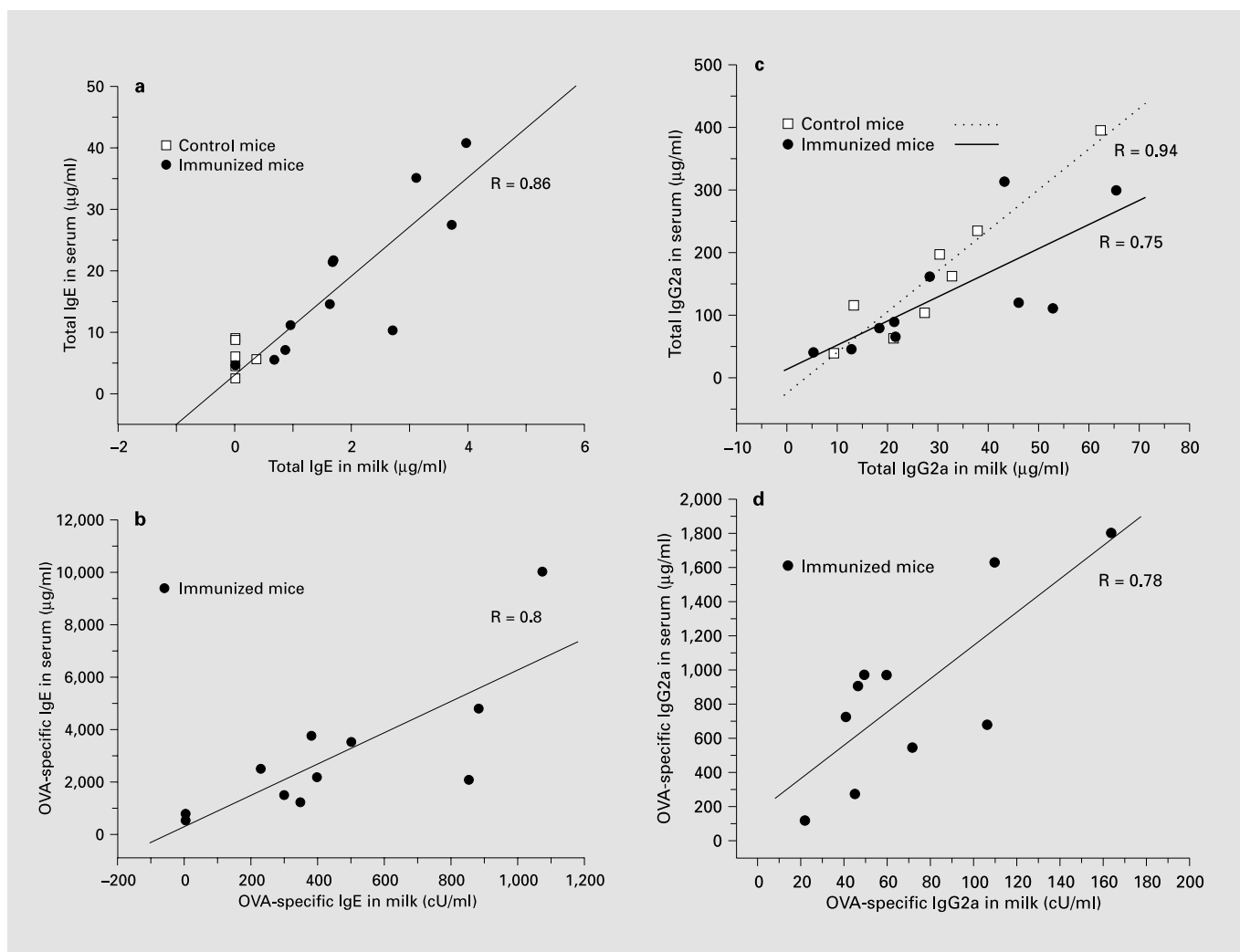


Fig. 3. Antibody levels in milk versus serum 1 week after delivery: total IgE (**a**), OVA-specific IgE (**b**), total IgG2a (**c**) and OVA-specific IgG2a (**d**). cU = Capture units.

IgE and IgG2a in serum and milk from immunized mice could be demonstrated, suggesting that the detected antibodies in milk originate from blood (fig. 3).

The Anti-OVA Antibody Response of the Pups

Immediately after birth the litters of the OVA-immunized and naïve mice were exchanged. At the age of 3 weeks all pups were given a single dose of OVA intraperitoneally and sacrificed 2 weeks later. Sera, spleens and lymph nodes were obtained and pooled for each litter. Serum was analyzed for total as well as OVA-specific IgE and IgG2a. The control group of pups born and nursed by naïve mothers (group A) showed the highest total IgE levels ranging from 0.8 to 1.2 µg IgE/ml. Total IgE levels in

the two remaining groups ranged from 0.2 to 0.36 µg IgE/ml. OVA-specific IgE could be detected only in pups, which were born and nursed by naïve mothers. Thus a significant suppression of the active IgE response of pups born or nursed by immunized mothers was observed (table 1). Neither total nor OVA-specific IgG2a levels showed significant differences between the three different groups (table 1). In our experimental setup this excludes the maternal influence on the IgG2a titers of the pups.

The Proliferative Response towards OVA

The OVA-specific proliferative responses of spleen and mesenteric lymph node cells obtained from the pups were analyzed by lymphocyte stimulation test using the

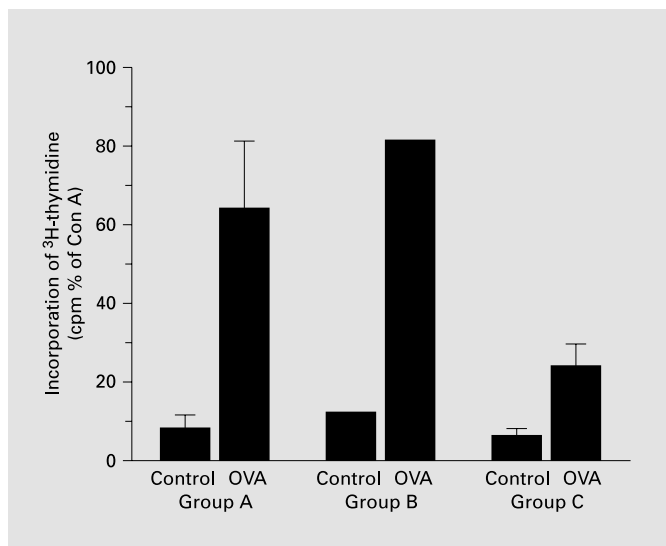


Fig. 4. Suppression of the specific proliferative response of mesenteric lymph node cells to OVA by milk of OVA-immunized mothers (group A: 11 pups from 4 litters; group B: 3 pups from 1 litter; group C: 8 pups from 2 litters; mean litter values $\pm$ SD).

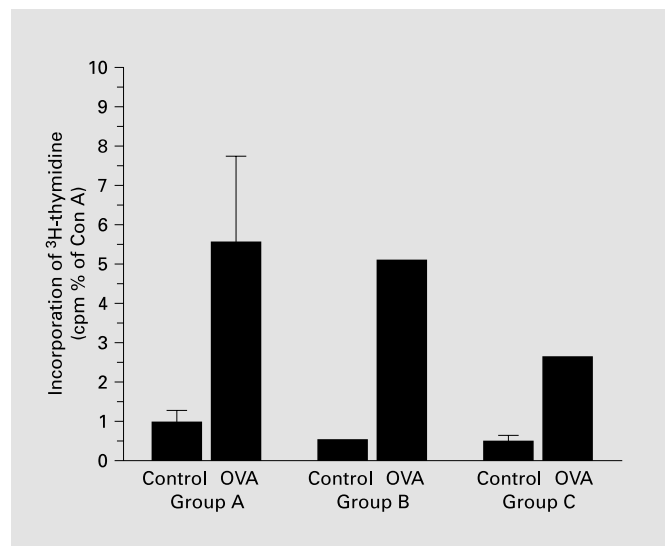


Fig. 5. Suppression of the specific proliferative response of spleen MNC to OVA by milk of OVA-immunized mothers (group A: 19 pups from 6 litters; group B: 3 pups from 1 litter; group C: 8 pups from 2 litters; mean litter values $\pm$ SD).

Table 1. Influence of maternal antigen exposure on the IgE and IgG2a production of the pups

	Number of litters	Number of pups	IgE		IgG2a	
			total μ g/ml	specific cU/ml	total μ g/ml	specific cU/ml
<i>Group A</i>						
Pups born to naïve mice nursed by their own mothers	3	12	1.01 $\pm$ 0.16	685 $\pm$ 173	82.2 $\pm$ 19.3	139.8 $\pm$ 14.1
<i>Group B</i>						
Pups born to immunized mice nursed by naïve foster mothers	2	8	0.33 $\pm$ 0.03	<0.8	39 $\pm$ 5.7	163.2 $\pm$ 13
<i>Group C</i>						
Pups born to naïve mice nursed by immunized foster mothers	2	10	0.24 $\pm$ 0.04	<0.8	43.2 $\pm$ 20.9	232 $\pm$ 78.5

The total and anti-OVA-IgE response of pups was significantly suppressed by OVA-immunized mothers either via breast-feeding or transplacentally (both $p \leq 0.05$). The total and specific IgG2a levels did not differ statistically between the three groups studied (Student's *t* test). cU = Capture units.

incorporation of ^3H -thymidine. As shown in figure 4, the proliferative response of mesenteric lymph node cells was significantly decreased in group C, naïve pups which were nursed by immunized mothers as compared to control pups (group A). The observed effect was statistically significant ($p \leq 0.01$, Student's *t* test), indicating that the

suppressed proliferation is caused by the factors transmitted in milk. Also when comparing the OVA-induced proliferative response of splenocytes (fig. 5) of the naïve pups fed by their own mothers (group A) with that of the naïve pups nursed by immunized mothers (group C), a clear and statistically significant suppression of T cell proliferation

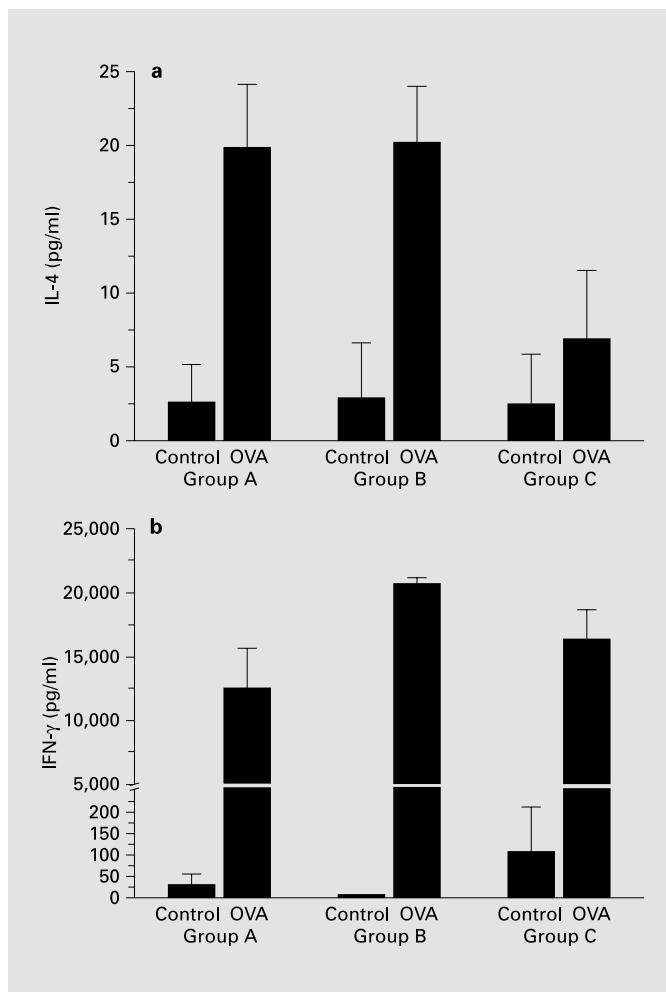


Fig. 6. **a** Suppression of the in vitro IL-4 production of spleen MNC to OVA by exposure to milk of OVA-immunized mothers during breast-feeding. **b** IFN- γ production was not statistically different between the three groups. Results are mean levels of cytokines from two individual assays $\pm$ SD.

in response to OVA could be demonstrated ($p \leq 0.02$, Student's *t* test).

In conclusion, the results show the suppressive effect of breast milk on the proliferative responses of the offspring's mesenteric lymph node and spleen cells upon stimulation with the specific antigen.

IL-4 and IFN- γ Production upon Stimulation with OVA

In order to further substantiate the suppression of Th2 responses observed in IgE production and lymphocyte proliferation on the molecular level, IL-4 and IFN- γ production by the pups' splenocytes upon stimulation with

OVA was measured (fig. 6a, b). The production of IL-4 was significantly suppressed in the spleen cells of naïve pups fed by immunized mothers ($p \leq 0.001$, Student's *t* test). Thus, similar to the proliferative responses, IL-4 production was significantly suppressed by factors transferred during breast-feeding. However, currently we cannot state whether individual T cells produce less IL-4 or there are less IL-4-producing T cells in the culture.

The IFN- γ production upon OVA stimulation of splenocytes was not statistically different between the three experimental groups. The pattern of IL-4 and IFN- γ production indicates a suppression of the Th2 but not Th1 cytokine response to OVA by exposure to milk of OVA-immunized mothers during breast-feeding.

Discussion

Several reports have shown that prolonged breast-feeding is prophylactic for atopic diseases [8, 20, 21], whereas other investigations could not confirm such a protective effect of breast-feeding [22, 23].

Atopic mothers, in contrast, were reported to modify the development of the infant's immune response towards the allergic phenotype [10, 11]. However, this was attributed mainly to transplacental exposure to maternal antibodies and cytokines. Additionally, Böttcher et al. [24] recently demonstrated that the concentrations of IL-4, IL-5 and IL-13 were higher in colostrum from allergic compared to nonallergic mothers. These cytokines may bias the immune responses of the breast-fed infants towards the Th2 phenotype. Hence allergic mothers may contribute to the development of allergy in their children by breast-feeding.

Here we could demonstrate, for the first time, the presence of substantial amounts of OVA-specific IgE in the milk of immunized mice. Based on these findings, one may suggest that specific antiallergen IgE could be an additional potential 'allergy' risk factor present in milk of atopic mothers. If transferred to the offspring, milk-borne IgE with common allergen specificity may give the immune system of the infant the first push towards the development of allergy. This may be happening by allowing for high efficiency allergen capture and presentation through CD23 on antigen-presenting cells [25, 26]. Such a mechanism was suggested to play a role in the broadening of the allergen response in atopics and the advancement of the disease from a 'single allergy' to a 'multiallergy' syndrome [26].

However, as we show here, the proallergic systems in milk do not lead to the transfer of the Th2 bias from mother to the offspring, at least not in our experimental setup. In contrast, a clear suppression of the primary specific IgE response of the infant mice by the milk of OVA-immunized mothers was observed. Our findings confirm earlier studies by Jarrett and Hall [27] and Roberts and Turner [28] where the selective suppression of IgE antibody responsiveness of newborn rats by maternal influence was illustrated. The mechanism of suppression of IgE production in young mice nursed by immune mothers still remains to be elucidated. Jarrett and Hall [29] drew attention to the possibility that maternal IgG in milk could be responsible for the suppression of specific IgE. This is in agreement with recent theories that balance of IgE and IgG determines the development of an atopic disease [30]. Accordingly, in our analysis we found high levels of OVA-specific IgG2a in the milk of immunized mice, which may counteract the effects of OVA-specific milk-borne IgE.

Additionally, the IgE/IgG balance in milk may be further altered by the differences in their uptake by the breast-fed infant. Maternal milk-borne IgG is actively transferred to the newborn mice through the MCH I-like receptor FcRn found on gut epithelial cells of murine neonates [31], which was recently also found on human gut epithelial cells [32]. So far there is no evidence for the transfer through the neonates' gut barrier of other immunoglobulin classes, including IgE.

Dannaeus and coworkers [33, 34] found that the levels of cow milk and egg protein-specific IgG correlate well between the sera of the human newborns and their mothers and provided evidence for serum IgE suppression by IgG. Additionally they showed that atopic mothers transfer larger amounts of specific IgG to their offspring than healthy mothers.

In the current study, in addition to the suppressed IgE response in pups fed by OVA-immunized mothers, we demonstrated the decreased specific proliferative response of their lymph node cells and splenocytes. Furthermore, we showed a decrease of IL-4 production *in vitro* by the splenocytes of pups nursed by immunized mothers upon stimulation with OVA. The IFN- γ production by the pups' splenocytes was not altered by maternal influence.

Adkins and coworkers [35–37] found that, in contrast to adult animals, neonates are biased to the Th2 function at all phases of an immune response and that they are selectively impaired in the development of a Th1 memory response. They state that environmental as well as cell

intrinsic influences may contribute to the Th2 skewing in neonates [38].

We showed that 'factors' transferred from immunized mothers via milk to the newborns may, however, direct the immune response of immunized young animals towards a Th1 phenotype and may, therefore, play a role in circumventing the onset of 'allergic' diseases.

Within the context of atopy the role of antibodies transferred via milk has been discussed in detail, yet the importance of the transfer of lymphocytes from mother to child via milk still remains to be elucidated. For example, Böttcher et al. [39] found elevated IL-8 and RANTES levels in colostrum from allergic mothers compared to non-allergic mothers, chemokines which may contribute to recruiting specific cell populations into breast milk.

In summary, we have demonstrated the suppression of the pups' primary humoral and cellular immune response towards OVA when breast-fed by mothers exposed to OVA shortly before the pregnancy. It appears that the transfer of the suppressive activity from mother to pups, mediated by breast milk, directs the pups' immune response away from the Th2 type, thus avoiding the 'allergic' phenotype.

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