Published in final edited form as:

FASEB J. 2007 April ; 21(4): 1264–1270. doi:10.1096/fj.06-7223com.

Anti-ulcer treatment during pregnancy induces food allergy in mouse mothers and a Th2-bias in their offspring

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Abstract

The treatment of dyspeptic disorders with anti-acids leads to an increased risk of sensitization against food allergens. As these drugs are taken by 30–50% of pregnant women due to reflux and heartburn, we aimed here to investigate the impact of maternal therapy with anti-acids on the immune response in the offspring in a murine model. Codfish extract as model allergen was fed with or without sucralfate, an anti-acid drug, to pregnant BALB/c mice during pregnancy and lactation. These mothers developed a codfish-specific allergic response shown as high IgG1 and IgE antibody levels and positive skin tests. In the next step we analyzed whether this maternal sensitization impacts a subsequent sensitization in the offspring. Indeed, in stimulated splenocytes of these offspring we found a relative Th2-dominance, because the Th1- and T-regulatory cytokines were significantly suppressed. Our data provide evidence that the anti-acid drug sucralfate supports sensitization against food in pregnant mice and favors a Th2-milieu in their offspring. From these results we propose that anti-acid treatment during pregnancy could be responsible for the increasing number of sensitizations against food allergens in young infants.

Keywords

sensitization against food; sucralfate; digestion; children; lactation

The incidence of food allergies has been steadily increasing in the past decades and these allergies now affect ~3.5–4% of the adult population in the United States(1) and ~2.2–5.5% of children in the first year of life (2). Apart from several environmental influence factors, no genuine explanation accounts for this high number of children with food sensitization. It is known that the risk for sensitization in the offspring is higher when one or both parents, but especially when the mother is atopic (3). This may be due to hereditary factors, but an atopic mother may also transfer factors that directly bias the immune response of the infant to an allergic phenotype.

For the adult population, we could show in previous studies that the risk to develop sensitization against food allergens increases with the usage of acid-suppressing drugs (4, 5), which are applied for the treatment of dyspeptic disorders such as gastric reflux, heartburn, and gastritis. We suggested that the hindered peptic digestion due to the elevated pH in the stomach leaves bigger fragments of alimentary proteins. These proteins could then elicit allergy due to persistence of their native conformation.

Approximately two-thirds of pregnant women suffer from heartburn and reflux due to low esophageal sphincter pressure, which is caused by changes of the hormone status (6). For their treatment, Richter suggested a step-up algorithm beginning with lifestyle modifications and dietary changes. As the first-line medical treatment antacids and sucralfate are used (7). The same review states that actually ~30–50% of women use antacids to relieve heartburn and other acid-reflux symptoms during pregnancy. For sucralfate, another nonabsorbable drug like antacids, only one randomized, controlled study exists investigating effects during pregnancy (8). No maternal or fetal adverse effects were reported, except diarrhea in 1 patient out of 42. However, it is known that sucralfate forms stable complexes with proteins, thereby inhibiting their hydrolysis by preventing pepsin/substrate interaction (9). Furthermore, sucralfate also blocks peptic activities by buffering gastric acid (10), and it directly adsorbs pepsin and bile salts. Therefore, we considered that these drugs can favor food sensitization also during pregnancy, which has never been tested before. More important, we worried that this sensitization impacts the development of allergic immune responses in the newborn. To test this hypothesis, we have chosen codfish extract as a model allergen, which was fed in combination with sucralfate to mother mice during pregnancy and lactation. Subsequently, immune responses were monitored on the humoral as well as on the cellular level in mothers and offspring.

MATERIALS AND METHODS

Preparation of codfish extract

Commercially available frozen codfish was used to prepare an extract, as described previously (11). The protein concentration of the extract was determined according to the method of Bradford (12) by using a Bio-Rad Protein Assay (Bio-Rad, Munich, Germany).

Protocol of sensitization

BALB/c mice (female, 6–8 wk; male, 6 wk) were purchased from Harlan Winkelmann GmbH (Borchen, Germany), acclimated for 7 d and treated according to European Community rules of animal care (13) with the permission of the Austrian Ministry of Science (number GZ 66.009/0033-BrGT/2005). Mice were mated, and the appearance of a vaginal plaque was considered day 1 of pregnancy. On days 15 and 19 (during pregnancy) and on days 32 and 45 (during lactation), mice were immunized intragastrically with either PBS (110 µl), codfish extract (2 mg in 110 µl PBS), or codfish extract (2 mg in 110 µl PBS) plus 2 mg sucralfate (Ulcogant, Merck, Vienna, Austria). Blood samples were drawn on days 2 (preimmune serum), 18, 25, 39, 53, and 59. Offspring of all mothers were immunized intraperitoneally (i.p.) on day 45 of age with 150 µg codfish extract mixed with 30 µg ovalbumin (OVA; Sigma, Darmstadt, Germany) in 50 µl PBS plus 2 mg aluminum hydroxide (Inject[®] Alum, Pierce, Rockford, IL, USA). Booster immunization was performed on day 58 of age with the same amounts of allergens but without aluminum hydroxide. Blood samples were collected on days 19, 32, 40, 52, and 66 of age.

Antibody detection in serum by enzyme-linked immunosorbent assays (ELISA)

Microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with codfish extract (5 µg per well in 100 µl 50 mM NaHCO₃, pH 9.6). Samples were diluted 1:100 for IgG1 and

IgG2a or 1:10 for IgE. Further ELISA procedure was performed as described previously (14).

Beta-hexosaminidase secretion assay from rat-basophile leukemia (RBL-2H3) cells

RBL-2H3 cells (generously provided by Dr. Arnulf Hartl, Institute of Biochemistry, University of Salzburg, Austria) were cultured in RPMI medium (Life Technologies, Inc.[®] Invitrogen, Lofer, Austria), supplemented with 10% fetal calf serum, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES, 1% penicillin/streptomycin, and 100 μ M 2-mercaptoethanol at 37°C and 5% CO₂. RBL cells (4×10^4 cells/well in 100 μ l medium) were seeded in a 96-well culture plate (96 MicroWell, Nunclon, Nunc, Roskilde, Denmark) and incubated overnight at 37°C and 5% CO₂. Cells were passively sensitized with pooled sera from mice (5 μ l/well) for 2 h at 37°C and 5% CO₂. Afterward, cells were washed twice with Tyrode's buffer (137 mM NaCl, 2.68 mM KCl, 1.4 mM CaCl₂, 1.7 mM MgCl₂, 5 mM D-glucose, 10 mM HEPES, 0.42 mM NaH₂PO₄×H₂O, 12 mM NaHCO₃) at pH 7.2, supplemented with 0.1% BSA (Sigma, Steinheim, Germany). Codfish extract or hazelnut extract (as irrelevant control allergen) were diluted in Tyrode's buffer and added to the wells (0.1 μ g in 100 μ l/well) for 30 min at 37°C and 5% CO₂. For negative controls, RBL-cells were incubated with Tyrode's buffer without allergens to calculate spontaneous release. Cell lysis was performed by addition of 10 μ l of 10% Triton-X (Sigma) in Tyrode's buffer at 37°C for 5 min for total release of β -hexosaminidase (100% release). Of the supernatants, 50 μ l was discarded and instead 50 μ l of assay solution (5 ml 0.1 M citric acid, pH 4.5, with NaOH, mixed with 80 μ l 4-methylumbelliferyl-N-acetyl-beta-D-glucosaminide; Sigma) at pH 4.5 was added. Cells were incubated for 1 h at 37°C and 5% CO₂, and the reaction was terminated by addition of 100 μ l glycine buffer (200 mM glycine, 200 mM NaCl, pH 10.7). Finally, the fluorescence was read at 360/465 nm, and results were calculated as percentage of total release.

Skin tests of mice

On the day of sacrifice, Evans blue (100 μ l of 5 mg/mL; Merck, Darmstadt, Germany) was injected into the tail vein of mice. Subsequently, 30 μ l of codfish extract (50 μ g/mL), recombinant birch pollen allergen Bet v 1 (2.5 μ g/mL; Biomay, Vienna, Austria) as irrelevant control allergen, mast cell degranulation compound 48/80 (20 μ g/mL; Sigma, Steinheim, Germany) as positive control, or PBS as negative control were administered intradermally into the shaved abdominal skin. After 20 min, mice were killed and skinned. A positive response is seen as a blue color reaction on the inside of the abdominal skin due to vascular leakage.

Isolation of splenocytes and detection of cytokines

Mice were sacrificed, and their spleens were removed. Spleen cell suspensions were prepared immediately by cutting, mincing, and filtering through 70 μ m nylon meshes. Cells were resuspended in RPMI medium, supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin. Mononuclear cells were isolated by density separation (Lympholyte[®]-M, Cedarlane, Ontario, Canada) according to manufacturer's instructions. Cells were plated (4×10^6 cells/ml) in sterile round-bottom 24-well tissue culture plates (Costar, NY, USA). For stimulation, codfish extract (500 μ g/ml), OVA (500 μ g/ml), recombinant Bet v 1 as control allergen (40 μ g/ml), concanavalin A (Con A) (4 μ g/ml; Sigma) as positive control, or medium were added. The cells were cultured for 4 d at 37°C and 5% CO₂. Supernatants were harvested and stored at -20°C until further use for cytokine measurements.

Measurement of IL-4, IL-5, IL-10, and IFN- γ was performed by ELISA with anti-mouse cytokine-antibodies and standards (eBioscience, San Diego, CA, USA) in undiluted supernatants of stimulated splenocytes, according to manufacturer's instructions.

Statistical analysis

Statistical comparison between groups was performed by the Mann Whitney *U* test, using the software statistical Packages for the Social Sciences (SPSS) (version 12.0 for Windows). Differences were considered statistically significant at *P* values <0.05.

RESULTS

Treatment of pregnant mice with sucralfate results in sensitization

Female BALB/c mice were fed twice during their late pregnancy (days 15 and 19 after conception) and twice during the lactation period (days 32 and 45 after conception, *i.e.*, 11 and 24 d after giving birth) with PBS, with codfish extract alone or with codfish extract together with sucralfate. After three gavages, mothers treated with sucralfate showed increasing IgG1-levels against codfish, which further increased after the fourth feeding (Fig. 1A). The presence of biologically relevant codfish-specific IgE at this time point was shown in an RBL-assay again only in the sucralfate-treated group (Table 1). The induction of an allergic response via sucralfate could further be confirmed in type I skin tests, where only mice treated with sucralfate showed a positive reaction to codfish, but mice fed with PBS or codfish alone did not (Fig. 1B).

Codfish-specific IgG1 from sucralfate-treated pregnant mice is transferred to the offspring

To evaluate the influence of the sensitization of mother mice described above, the antibody levels in sera of the offspring of these animals were monitored over a period of more than 60 d. The first evaluation was performed in serum obtained from offspring at day 19 of age without any intervention. Significant levels of codfish-specific IgG1 could only be found in the offspring from mothers treated with sucralfate (Fig. 2). No other subclasses of codfish-specific antibodies or antibodies to OVA were found in any of the groups at this time point (data not shown).

Specific IgE to homologous codfish antigen is suppressed in sera of immunized offspring of sucralfate-treated mother mice

The impact of maternal codfish-specific sensitization on the offspring was investigated by immunizing the young animals twice *i.p.* with a mixture of the homologous codfish antigen and the heterologous protein OVA, adsorbed to aluminum hydroxide. Regarding antibody titers for codfish- or OVA-specific IgG1 (Fig. 3A) and IgG2a (data not shown), no significant difference was observed between the offspring of the differently treated mother mice at the age of 66 d. However, analyzing codfish-specific IgE levels after two *i.p.* immunizations, we found a significant suppression only in offspring of sucralfate-treated mice (Fig. 3B). This suppression was allergen-specific, as no influence on OVA-IgE was revealed.

The allergen-specific suppression of IgE in serum of offspring was confirmed by RBL-assay (Fig. 4). No significant differences between the various offspring groups could be seen on the levels of total IgE at this time point (data not shown).

Generation of IFN- γ and IL-10 is suppressed in splenocytes of offspring from sucralfate-treated mice

In contrast to the antigen-specific suppression of IgE at the humoral level, the results of the cytokine analysis showed a significant suppression of IFN- γ and IL-10 after stimulation with both, codfish or OVA, suggesting an antigen-independent influence on T cell activation in offspring of sucralfate-treated mother mice (Fig. 5). However, the Th2-cytokine IL-5 was not altered in the young mice of sucralfate-treated mothers compared to the other groups (data not shown). IL-4 could not be detected in supernatants of any group.

DISCUSSION

We have shown previously that anti-acid treatment is an important factor for induction of food-specific IgE in adult humans (5) and in mouse models (4, 11). These anti-acid medications are also used as first-line drug treatment for up to 50% of pregnant women (15). The aluminum-containing drug sucralfate as well as antacids are considered to be safe during gravidity for the fetus, because none of these drugs is absorbed systemically (16). However, we hypothesized that sensitization to food could occur when consuming those drugs during pregnancy. The present study was designed to evaluate the influence of such treatment on the immune response of the pregnant/lactating mother and their offspring.

In a BALB/c mouse model, it became obvious that intragastric immunizations of pregnant mothers in the third trimester and during lactation induce codfish-specific IgE and IgG1 as well as positive skin tests when codfish protein was fed in context with sucralfate. The underlying mechanism may be a hindrance of the peptic digestion through acid neutralization or pepsin binding. Consequently, bigger peptide fragments or even remnants of proteins can enter the gastrointestinal tract and lead to the induction of specific IgE (17).

Such an allergic status of the mother has the potency to affect the immune response of the offspring by numerous factors transferable via placenta or breast milk. For instance, a trans-amniotic transfer of intact maternal IgE into the amniotic fluid can occur (18); maternal DNA was found in cord blood (19); trafficking of leukocytes from mother to fetus *in utero* has been shown (20); and chemokines, like IL-8, RANTES, IP-10, or MIG, were detected in breast milk (21, 22). Furthermore, the transfer of allergens (23–27) as well as antibodies (28, 29) via placenta or breast milk has long since been recognized. Also in our study, high codfish-specific IgG1 antibodies were found in the offspring of the mice treated with sucralfate. This specific IgG1 in the naive offspring was most likely of maternal origin.

The impact of this humoral immune response on a subsequent sensitization was investigated by immunizing the young animals on the one hand with the homologous antigen codfish, which the mother was given, and on the other hand to a foreign heterologous antigen, which neither the mother nor the offspring has encountered before. Interestingly, when high IgG1-antibody levels were found in the offspring, the generation of codfish-specific IgE-antibodies was significantly suppressed. This phenomenon has already been observed previously (29, 30). A possible explanation is that maternal IgG1 can function as blocking antibodies via formation of immune complexes, which then attach to the Fc γ R2-receptor and the B-cell receptor on B-cells, generating negative intracellular signals by cross-aggregation of these receptors (30, 31).

Interestingly, in splenocytes of young animals from sucralfate-treated mothers we found a dominating Th2-cytokine milieu, characterized by significantly reduced levels of IFN- γ and IL-10 but unchanged levels of IL-5 after restimulation with the homologous antigen codfish. Generally, at the time of birth, IFN- γ levels are lower compared with adults (32), which may be due to the Th2-milieu necessary for a successful pregnancy (33). Our data confirm

previous observations that the offspring of sensitized mothers are prone to experience a further suppression and a later onset of normal levels of the Th1-cytokine IFN- γ due to a lower frequency of IFN- γ -producing cells (30). A dominance of a Th2-response also to a heterologous antigen was found by this working group. Accordingly, after stimulation with OVA being a heterologous antigen, we revealed that Th1-cytokines were significantly suppressed, resulting in a dominance of the general Th2-cytokine response, although no elevation of Th2-dependent OVA-antibodies was observed.

The production of IL-10 was also significantly suppressed in offspring of sucralfate-treated mothers, indicating a diminished presence of or production by T-regulatory cells. The development of allergen-specific T-regulatory cells appears to be a critical event in the control of a balanced immune response to allergens and IL-10 by its immune modulating and antiinflammatory effects occupies a crucial role in this process (34, 35). Therefore, in our model the offspring not only showed a suppressed Th1-response and a resulting dominance of the Th2-branch, but simultaneously a lack of immune-modulating cytokines of T-regulatory cells.

We are aware of the fact that the suppression of specific IgE and the concomitant general Th2-shift are somehow contradictory. However, previous murine studies support our observations: There is a preventive effect of a specific sensitization of the mother against the respective antigen in the offspring via IgG-antibodies. At the same time, other factors transported from the mother may be responsible for a broad Th2-response toward multiple heterologous antigens in the newborns (28, 30, 31). The present study shows for the first time that the same effects can be achieved by the oral route when sucralfate is taken. From our data, we thus conclude that factors like sucralfate, supporting the Th2-environment around birth, could enhance the risk of allergic sensitization in the child. However, this has to be reinvestigated in the human system in the future.

Our data provide the first evidence that the intake of sucralfate during pregnancy leads to specific antibody formation against food allergens in the pregnant mother. This sensitization influences the developing immune system of the offspring by inducing a shift toward a Th2-dominated milieu, increasing the risk for allergic sensitization in these individuals. These mechanisms may explain the rising number of food allergy in children during the past decades, which parallels the increasing consumption of antiulcer drugs during pregnancy.

Acknowledgments

We thank Anja Spies-Naumann, Anka Wensing, Stefanie Achenbach, Thomas Ruppertsberg, and Brigitte Auffarth for their excellent technical assistance. I.S. was supported by a Hertha-Firnberg stipendium (T283-B13), E.U. by a Charlotte-Bühler stipendium (H220-B13) and work was supported by SFBF018#8 from the Austrian Science Fund (Vienna, Austria), and I.S. was further supported by a research fellowship from the Alexander-von-Humboldt Foundation (Bonn, Germany).

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Figure 1.

Sucralfate induces sensitization against concomitantly applied codfish proteins. *A*) Intragastric application (arrows) of codfish extract in context with the antiulcer drug sucralfate (black symbols) to mother mice ($n=6$ per group) during pregnancy, and lactation leads to increasing codfish-specific IgG1. Control groups of pregnant mice were fed with either PBS (white symbols) or codfish extract alone (gray symbols). The gray area marks the lactation period. Mean values of groups are shown. *B*) Sucralfate-treatment also induced a positive reaction to codfish in type I skin tests of mother mice ($n=6$ per group). No reaction to codfish was observed in mothers fed with either PBS or codfish extract alone, respectively to control substances (allergen Bet v 1 from birch pollen or PBS). Substance 48/80 was used as positive control. Representative skin tests of each group are shown.

Figure 2.

Codfish-specific maternal IgG1 antibodies are transferred to the offspring. In naive offspring of mother mice treated with sucalfate during pregnancy and lactation, significant amounts of codfish-specific IgG1 could be detected in sera 19 d after birth (black column, $n=20$). No codfish-specific antibodies were found in the offspring of mothers treated with PBS (white column, $n=12$) or codfish extract alone (gray column, $n=10$). Data are representative for two independent experiments and expressed as mean + SD (***) $P<0.001$).

Figure 3.

A significant suppression of codfish-specific IgE was found in offspring of sucralfate-treated mice. Offspring were immunized i.p. twice simultaneously with the homologous antigen codfish mixed with the heterologous antigen OVA (arrows). Only in the offspring from sucralfate-treated mothers (black symbols, $n=20$), the generation of codfish-specific IgE but not OVA-specific IgE was significantly suppressed (B). No influence could be found in the offspring of mice treated with PBS (white symbols, $n=12$) or codfish extract alone (gray symbols, $n=10$), or on the levels of codfish- or OVA-specific IgG1 (A) or IgG2a (data not shown). Data are representative for two independent experiments and expressed as mean $\pm$ SE (* $P<0.05$).

Figure 4.

The suppression of codfish-specific IgE was confirmed by β -hexosaminidase release of RBL cells. Sera of offspring before and after two i.p. immunizations (from days 19 and 66 after birth, respectively) were investigated. After passive sensitization of RBL cells with pooled sera of offspring and stimulation with codfish extract, the release of hexosaminidase was much lower in the group where mother mice were treated with sucalfate (black symbols) than in groups of treatment with PBS (white symbols) or codfish (gray symbols). Mean values of triplicates of codfish-induced release of β -hexosaminidase as percentage of total release (after lysis of cells) are shown. Due to the usage of pooled sera statistical analysis could not be performed.

Figure 5.

Sensitization of mothers leads to an antigen-independent suppression of IFN- γ and IL-10 production by spleen cells from offspring. Splenocytes of offspring were stimulated with the homologous allergen codfish and the heterologous antigen OVA. A significant suppression of the Th1-cytokine IFN- γ and the T-regulatory cytokine IL-10 in the offspring of mothers treated with codfish plus sucralfate (black columns, $n=20$) compared with PBS-treated (white columns, $n=12$) or codfish-treated mothers (gray columns, $n=10$) was observed. In contrast, no difference was found in the levels of the Th2-cytokine IL-5 (data not shown). Mean values of groups $\pm$ SD representative for two independent experiments are shown (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). The bottom panel indicates that the ratio of the Th2-cytokine IL-5 compared with the suppressed Th1-cytokine IFN- γ was highest in offspring of sucralfate-treated mother mice. This was seen after stimulation of splenocytes with codfish as well as with OVA.

TABLE 1

Codfish-specific IgE in pregnant mice is induced after concomitant feeding of sucralfate

Passive sensitization with serum of mothers treated with	<u>Stimulation of RBL-cells</u>	
	Codfish extract	Hazelnut extract
PBS	8.9%	9.9%
codfish extract	9.3%	9.1%
codfish extract + sucralfate	16.9%	9.6%

Numbers give means of triplicates of β -hexosaminidase release as percentage of total release with Triton-X[®] (=100%; negative controls with medium or buffer gave 8.5% release). Measurement of fluoro-metrical intensity (360/465 nm) was performed. No statistical analysis could be performed due to the usage of pooled sera.