A model of chronic IgE-mediated food allergy in ovalbumin-sensitized mice

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Abstract

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Food allergy is most frequently the result of IgE-mediated hypersensitivity reactions. Here, we describe a chronic model in which some of the intestinal and systemic consequences of continuous egg white solution ingestion by ovalbumin-sensitized eight-week-old BALB/c mice, 6 animals per group, of both sexes, were investigated. There was a 20% loss of body weight that began one week after antigen exposure and persisted throughout the experiment (3 weeks). The sensitization procedure induced the production of anti-ovalbumin IgG1 and IgE, which were enhanced by oral antigen exposure (129% for IgG1 and 164% for IgE, compared to sensitization values). Intestinal changes were determined by jejunum edema at 6 h (45% Evans blue extravasation) and by a significant eosinophil infiltration with a peak at 48 h. By day 21 of continuous antigen exposure, histological findings were mild, with mast cell hyperplasia (100%) and increased mucus production (483%). Altogether, our data clearly demonstrate that, although immune stimulation was persistently occurring in response to continuous oral antigen exposure, regulatory mechanisms were occurring in the intestinal mucosa, preventing overt pathology. The experimental model described here reproduces the clinical and pathological changes of mild chronic food allergy and may be useful for mechanistic studies of this common clinical condition.

Introduction

The physiological penetration of dietary macromolecules into the organism through the gut mucosa has several recognized immunological consequences. One of these is the development of food allergy, which is defined as an adverse immunological (hypersensitivity) response to food and can be IgE dependent or not (1). IgE-dependent food-allergic reactions may affect one or more target organs: the skin (urticaria, angioedema), the respiratory tract (rhinitis, asthma), the gastrointestinal tract (pain, emesis, diarrhea), and the cardiovascular system (anaphylactic shock) (2).

Food allergy is particularly frequent during childhood and may affect children's final

height and weight (3). Food allergy and other types of allergies, such as allergic rhinitis and asthma, are reaching epidemic proportions in both the developed and developing countries (4). The reasons for the changes in prevalence of allergic disorders are not known and several hypotheses have been raised to explain them, including the "hygiene hypothesis", which states that a reduction in infections in early infancy predisposes to allergic responses (5,6). The understanding of regulatory mechanisms controlling allergic inflammation may provide useful insights into disease pathophysiology and aid in the development of novel therapeutic strategies.

We have reported that mice previously sensitized against ovalbumin (Ova) avoided the ingestion of a sweetened solution of egg white, choosing water instead (7,8), and this aversion was related to IgE antibodies (9). Indeed, elimination diet is still the mainstay of IgE-dependent food allergy therapy (2,3). However, with the advent of manufactured food, trace amounts of allergens can still be present in food and some atopic patients appear to be continuously exposed to these allergens. In these atopic patients, continuous unwilling exposure to a food allergen may induce a mild and persistent allergic condition. To try mimicking the latter clinical situation, we have developed an experimental model in which sensitized mice are given egg white solution (EWS) as the only liquid source.

Material and Methods

Animals

Young adult BALB/c mice of both sexes were used. All mice received standard (Purina) mouse chow throughout the experiment. All investigations were in agreement with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation of our Institution (CETEA/UFMG).

Sensitization protocol

Mice received (day 0) a subcutaneous (*sc*) injection of 0.2 ml saline containing 10 µg Ova (five times crystallized hen's egg albumin; Sigma, St. Louis, MO, USA) plus 1 mg $Al(OH)_3$ as adjuvant. Secondary sensitization consisted of a *sc* injection of 10 µg soluble Ova 14 days after the primary sensitization (day 14). Although it has been shown that intraperitoneal injection of alum induces higher antibody production (10), we chose the *sc* route to avoid peritoneal inflammation. Seven days after the secondary sensitization (i.e., on day 21), the bottle containing tap water was replaced with gauze filtered 20% EWS for a period of 3 weeks (from day 21 to day 42). This solution contains about 10 mg Ova/ml. The following control groups were studied in parallel for comparison: non-sensitized or sensitized mice that were given tap water throughout the experimental procedure and non-sensitized mice that were given EWS from day 21 to day 42.

Nutritional status

In order to evaluate the nutritional condition of all animals, liquid and food consumption and body weight were determined each week. After 21 days of continuous EWS ingestion, hematocrit levels were determined and serum albumin levels were measured using an Albumin Doles Kit (Goiania, GO, Brazil) in order to assess the possible presence of dehydration and/or malnutrition.

Serum antibodies

Serum samples were obtained from groups culled weekly after oral antigen exposure for the measurement of anti-Ova IgG1 and IgE. The ELISA test for IgG1 was carried out using plates coated with Ova, 100 µl of a 1:1600 dilution of mouse sera and biotinylated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL,

USA). The reactions were developed with the streptavidin-peroxidase conjugate (ExtraAvidin; Sigma), o-phenylene-diamine and H_2O_2. The plates were read at 492 nm on an automated ELISA reader (EL800, Bio-Tek Instruments, Inc., Winooski, VT, USA). Anti-Ova IgE antibodies were measured by capture-ELISA using plates coated with rat anti-mouse IgE and 50 µl total serum and biotinylated Ova, as previously described (11). The results are reported as arbitrary units using a positive reference serum assigned to be 1000 units.

Intestinal edema

Vascular permeability changes were evaluated by Evans blue dye extravasation (12). Twenty-one days after the primary sensitization with 10 µg Ova + 1 mg $Al(OH)_3$, the animals were exposed to a restricted and voluntary intake of EWS for 6, 24 and 48 h and then injected with Evans blue (20 mg/kg) into the tail vein; 10 min later, the animals were killed and the small intestine was dissected and weighed. The intestine was divided into four segments (duodenum, proximal jejunum, distal jejunum, and ileum) and each segment was further divided into two parts. One was soaked in formamide (4 ml/g wet weight tissue at 20ºC for 24 h) to extract the Evans blue dye, and the other was dried at 60ºC for 24 h. The concentration of Evans blue was determined by spectrophotometry at 630 nm using an ELISA reader (EL800, Bio-Tek Instruments) and 96-well plates. Results were plotted on a standard curve for Evans blue (0.15-20 µg/ml). The Evans blue content of each sample is reported as mg/g dry weight tissue.

Histology and morphometry

Changes in the intestinal mucosa were evaluated at 6, 24 and 48 h, and at 7, 14 and 21 days of the experiment. The mice were sacrificed by cervical dislocation and the small intestine was taken for histological analysis. The intestine was divided into four segments (duodenum, proximal jejunum, distal jejunum, and ileum), fixed in 10% formalin in PBS, embedded in paraffin and cut into 3-5-µm thick sections. The sections were stained with hematoxylin-eosin for general analysis, with Toluidine blue for mast cell evaluation, with periodic acid Schiff for mucus analysis, or with 1% carbol chromotrope 2R and hematoxylin (Sigma) for eosinophil granule identification.

As described elsewhere, the intestinal mucosa was submitted to morphometric analysis using an image analysis program running on an IBM computer (13). Images were obtained with a JVC TK-1270/RGB microcamera and the KS300 software built in a Kontron Eletronick/Carl Zeiss image analyzer. Ten fields from hematoxylin-eosin-stained sections were randomly chosen at 10X (871.392 μm^2/field) for villus height and crypt depth measurement. Ten fields were chosen randomly at 40X (53.333 μm^2/field) from chromotrope and Toluidine blue sections in order to count the number of eosinophils and mast cells, respectively, and the data are reported as number of cells/field. For the determination of goblet cell volume, all pixels with green hues were selected for the creation of a binary image and subsequent calculation of the total area, and the data are reported as μm^2 mucus/field.

Statistical analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA) or by the Student t-test, when appropriate, with the level of significance set at $P < 0.05$. If ANOVA revealed differences, the groups were compared by the Student t-test with Bonferroni correction. The data shown in the Figures are reported as means ± SEM for 6 animals per group. In the text, the data are reported as means ± SD for 6 animals per group (N) or as the range for all groups.

Figure 1. Body weight of mice up to 42 days after the first sensitization with ovalbumin (Ova). Data are reported as means ± SEM in g for 6 mice in each group. BALB/c mice received (Ova⁺) or not (Ova⁻) 10 µg Ova plus $Al(OH)_3$ on day 0 and 10 µg Ova on day 14. From day 21 to the end, some Ova⁺ and Ova⁻ animals received filtered 20% (w/v) egg white solution in their drinking bottle (EWS⁺). *P < 0.05 compared to Ova⁻/EWS⁺ (ANOVA-Student *t*-test).
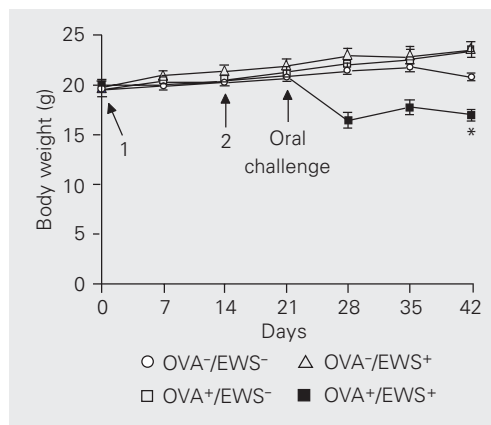

Figure 2. Anti-ovalbumin (Ova) IgG1 and IgE levels of mice sensitized with Ova. Serum antibody responses of BALB/c mice sensitized with 10 µg Ova plus $Al(OH)_3$ on day 0 and with 10 µg Ova on day 14. From day 21 to the end, some Ova⁺ and Ova⁻ animals received filtered 20% egg white solution in their drinking bottle (EWS⁺). Data are reported as means ± SEM of IgG1 and IgE expressed as arbitrary ELISA units for 6 animals per group. *P < 0.05 compared to Ova⁻/EWS⁻, and **P < 0.05 compared to Ova⁺/EWS⁻ (ANOVA-Student *t*-test).
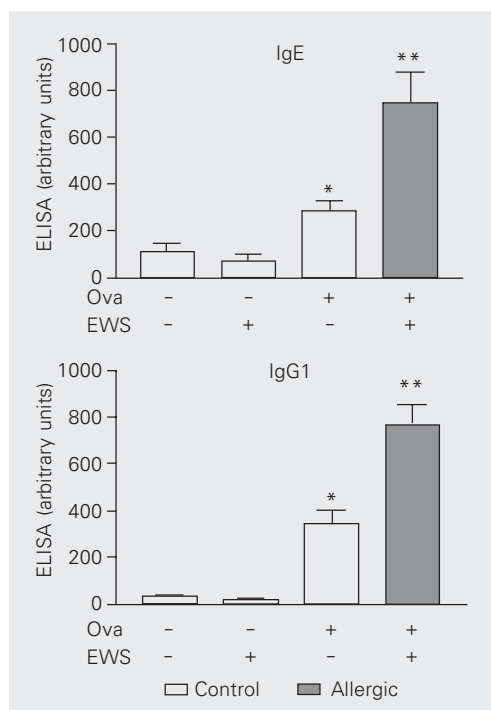

Figure 3. Intestinal vascular permeability. Extravasation of Evans blue dye in the small intestine (D - duodenum, PJ - proximal jejunum, DJ - distal jejunum, I - ileum) triggered by oral challenge with egg white solution (EWS) during a period of 6 h by sensitized (Ova⁺) (closed bars) and non-sensitized (Ova⁻) (open bars) mice. Animals were sensitized 21 days before the challenge by an injection of 10 µg OVA plus 1 mg $Al(OH)_3$. Data are reported as means ± SEM of Evans blue/g tissue for 6 animals per group. *P < 0.05 compared to control Ova⁻/EWS⁺ (ANOVA-Student *t*-test).
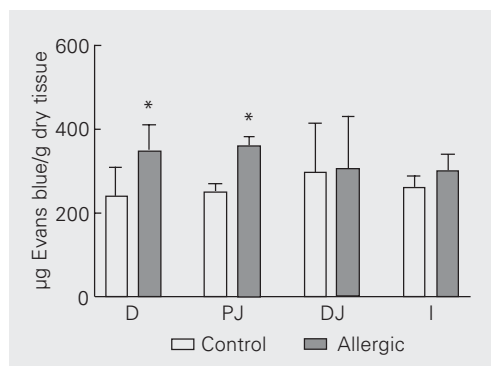
Results

The mean daily tap water consumption was similar for sensitized (4.7 ± 0.2 ml) and non-sensitized (4.2 ± 0.2 ml) mice, whereas the mean daily EWS consumption was higher for non-sensitized mice (8.3 ± 0.8 ml) compared to sensitized mice (4.1 ± 0.4 ml). All groups showed the same food consumption (data not shown). One week after the oral challenge with EWS, sensitized, but not non-sensitized, mice had lost about 20% of their body weight (Figure 1). The loss of body weight was maintained throughout the observation period and was not observed in animals who ingested tap water (Figure 1). The decreased body weight appeared not to be related to dehydration since there were no significant differences in hematocrit levels among groups (48-54%). Likewise, serum albumin levels were similar among groups before and after oral exposure to EWS or water (3.4-3.7 g/dl).

The sensitization procedure by itself induced the production of both anti-Ova IgG1 and IgE (Figure 2). Remarkably, the concentration of both of these antibodies more than doubled in sensitized mice that were given EWS solution, but not in sensitized mice given tap water (Figure 2). EWS consumption did not affect circulating levels of immunoglobulins in non-sensitized mice (Figure 2).

Twenty-one days after Ova sensitization, mice were exposed to restricted and voluntary EWS for 6 h. During this time, they ingested about 0.8 ml of the solution (8 mg Ova) and developed an increase in the vascular permeability of the proximal segments of the small intestine (Figure 3), as shown by the extravasation of Evans blue dye. There were no statistical differences in dye extravasation among the control groups at 6 h, or among all groups at later time points (data not shown). However, the early increase in vascular permeability generated villus swelling 24 h after EWS ingestion in sensitized mice (Figure 4B).

Inflammatory cell infiltration, with an

evident presence of eosinophils (Figure 4C), was seen as early as 6 h after antigen exposure. However, the number of eosinophils in the jejunal mucosa peaked at 48 h after antigen exposure and dropped thereafter (Figure 5A). Nevertheless, eosinophils were still present at significantly higher levels than in control animals until the end of the observation period (day 21).

With the continuous exposure to antigen (about 47 mg Ova/day), the pathological changes markedly improved and, at 21 days after oral antigen exposure, the intestine showed no edema and little infiltration of inflammatory cells other than eosinophils. However, there was increased mucus production by goblet cells (Figures 4D and 5B) and mast cell hyperplasia (Figures 4E and 5C) in the jejunum. The total mucosa height did not differ from control (Figure 5D).

No morphological differences were seen in any segment of the small intestine of non-sensitized mice given water or EWS or of sensitized mice given water when compared to the intestine of naive mice.
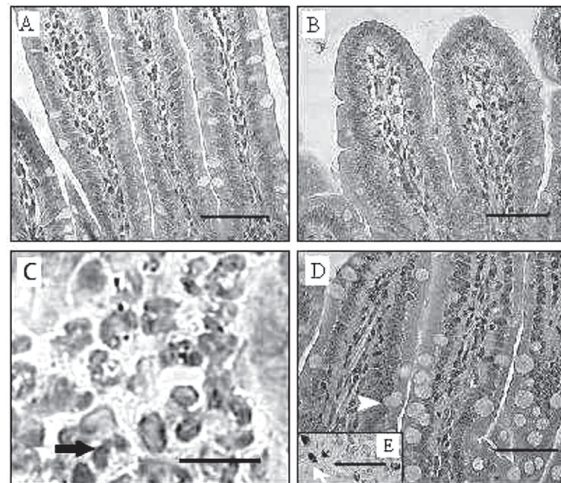

Figure 4. Representative histological sections of the proximal jejunum from non-sensitized mice drinking water (A), and from sensitized mice after 24 h (B), 48 h (C) and 21 days (D, E) of continuous ingestion of a filtered 20% egg white solution. A, B and D, hematoxylin and eosin staining; C, chromotrope 2R staining; E, Toluidine blue staining. The bars are 50 µm in A, B, D and E, and the bar in C is 25 µm. The large arrow in C indicates an eosinophil, the small white arrow in E indicates a mast cell, and the white arrowhead in D indicates a goblet cell.
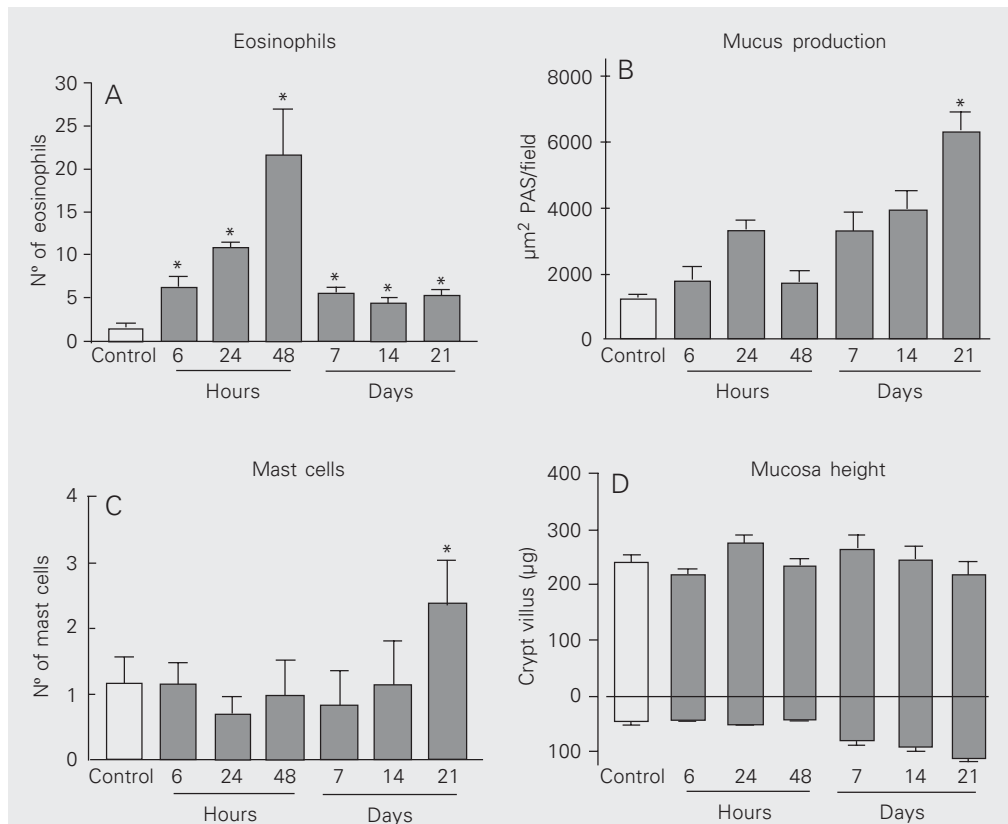

Figure 5. Eosinophils (A), mucus production (B), mast cells (C), and mucosa height (D) in the jejunum of BALB/c mice sensitized with 10 µg ovalbumin (OVA) plus 1 mg Al(OH)$_3$ after continuous ingestion of an egg white solution (EWS). The open bars indicate the control group (Ova⁻/EWS⁻) and the closed bars represent the allergic group (Ova⁺/EWS⁺) who ingested the EWS. Data are reported as means ± SEM of number of cells (A, C), mucus production (B; PAS = periodic acid Schiff), or mucosa height (D) for 6 mice in each group *P < 0.05 compared to control Ova⁻/EWS⁻ (ANOVA-Student t-test).

Discussion

There is much experimental evidence showing that the introduction of antigens via the gastrointestinal route induces oral tolerance, defined as the inhibition of specific antibody formation after subsequent parenteral immunizations with the same antigen (14). However, our results suggest that, when mice are sensitized to OVA, the subsequent long-term, low-level exposure to this same antigen via the oral route serves as an immunological booster leading to high levels of IgE and IgG1 production. Indirectly, these experiments suggest that a small portion of the ingested antigen is indeed capable of transposing the gut barrier and stimulating primed lymphocytes. Usually, IgE-dependent food allergy reactions affect one or more target organs such as skin, respiratory tract, gastrointestinal tract, and cardiovascular system (2). An immediate systemic reaction mediated by IgE is called anaphylaxis irrespective of severity (15), but colloquially the term anaphylaxis is applied to severe, potentially fatal allergic reactions (2). Mainly in children, chronic food allergic disorders (IgE-associated) typically affect the gastrointestinal tract with different degrees of eosinophilic inflammation, edema and weight loss (16).

Sensitized mice showed the same consumption of egg white and tap water, but the consumption of egg white by non-sensitized mice was higher than that by sensitized animals. The latter result suggests that there was an aversion to EWS by sensitized mice. This immunologically taste aversion was documented before (7,8) and was related to IgE responses (9). Recently, Basso and co-workers (17) have shown that after an oral Ova challenge, allergic mice presented higher levels of anxiety, increased Fos expression in emotionality related brain areas, and also aversion to Ova-containing solution. In our model, after prolonged egg white consumption, sensitized mice also showed a marked

loss in body weight. The mechanisms involved in this phenomenon are not clear, although this event has also been observed in rat models (18,19). Although the hematocrit and serum albumin levels were normal, suggesting that dehydration and/or malnutrition did not occur in mice with food allergy, we cannot exclude the possibility that a decreased absorption of other diet components or an increased energy consumption may have occurred. It is possible that the mechanisms involved in body weight loss are related to food aversion. By day 21, body weight was still low and circulating IgE and IgG1 levels were highest. These responses were related to the genetic background, since when another lineage was used (C57BL6), sensitization induced the production of IgE, but at lower levels when compared to BALB/c mice and there was no loss in body weight (data not shown). Also, our preliminary data have shown that IL-4 receptor alpha-deficient BALB/c mice do not produce IgE when immunized, nor do they lose body weight when challenged. These results indicate that this model is strongly related to Th2 cytokines.

After 6 h of antigen intake, vascular permeability was increased, as detected histologically at 24 h. This acute inflammatory change may be a result of local mast cell degranulation in response to Ova challenge and of the subsequent increase of histamine levels in the gut, as has observed elsewhere in sensitized mice after Ova challenge (20). In chronic allergic diseases such as asthma, during continuous antigen exposure eosinophils are primed by IL-5 and attracted by chemokines, infiltrating the tissue (21). These cells are responsible for the late phase of the immediate allergic reaction, producing basic protein which is toxic to the epithelium (21). Particularly in the jejunum, we observed that an eosinophil infiltrate was present as early as 6 h after exposure, with a peak at 48 h followed by low but significant levels until the end of the experiment.

With continuous exposure to the antigen,

pathological changes were marked by goblet cell and mast cell hyperplasia. Mast cell numbers in tissues are relatively constant, even though mast cell hyperplasia is observed in both the inflammatory and in the repair/remodeling stage of various inflammatory/fibrotic disorders (22). The functional significance of the accumulation of mast cells in these processes is largely unknown. In allergy, apart from their classical role in eliciting the early phase, mast cells also have an important function in late and chronic stages (23). In these stages they may interact with and be activated by infiltrated inflammatory cells such as eosinophils and lymphocytes and by resident structural cells such as epithelial and smooth muscle cells and fibroblasts.

Mucus hypersecretion is another characteristic of allergic inflammation and has been shown to be IL-13 dependent (24). It has been proposed that mucus protects the intestinal wall by limiting the absorption of antigens (25).

Taken together, our data clearly demonstrate that, although immune stimulation was persistently occurring in response to continuous oral antigen exposure, regulatory mechanisms in the intestinal mucosa prevented overt pathology. The mechanisms underlying the regulation of intestinal pathology in the model were not investigated here but clearly deserve further study. Moreover, it will be important to determine whether regulatory events triggered by chronic oral antigen exposure are antigen specific and restricted to the intestinal mucosa. The model of food allergy described here can be important to answer the questions raised above and may permit further understanding of the functional relevance of regulatory events triggered by chronic oral antigen exposure in allergic individuals.

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