

ORIGINAL ARTICLE

Murine model of food allergy after epicutaneous sensitization: Role of mucosal mast cell protease-1

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Abstract

Objective. Studies of the pathological mechanisms of food allergy have been impeded by the lack of relevant animal models. The purpose of this study was to develop a physiological model of food allergy that was not dependent on immunostimulatory adjuvants. **Material and methods.** Balb/c mice were epicutaneously sensitized four times at varying intervals over a 22-day period, and challenged orally from day 40, 6 times every 1–3 days with either saline or ovalbumin. **Results.** After sensitization (day 35) but before the oral challenges, the ovalbumin-sensitized groups showed increased specific IgE and IgG₁ production when compared with the sham-sensitized groups. Mucosal mast cell protease-1 (MMCP-1) was undetectable in serum before the intragastric challenge. MMCP-1 concentrations were increased after the first ovalbumin dose, solely in the ovalbumin-sensitized and -challenged group. After the challenge period, the mean serum MMCP-1 concentration increased from an undetectable level in controls to an over 44-fold level in the ovalbumin-sensitized and -challenged mice. In this group, MMCP-1-positive cells were present in the small intestine and expressions of IFN- γ and CXCL-9 mRNA were decreased in the ileum, suggesting an impaired Th-1-type response. Within one hour of the last ovalbumin challenge, 5 out of 6 mice developed diarrhea in the ovalbumin-sensitized and -challenged group, but there was no diarrhea in the other groups. **Conclusions.** A murine model of food allergy based on sensitization via epicutaneous exposure to allergen without immunostimulatory adjuvants was developed. Effective production of MMCP-1 together with specific IgE and IgG₁ suggests a breakdown in oral tolerance to the allergen. Intragastric challenges were accompanied by mast cell-dependent immunopathological changes and diarrhea.

Key Words: Diarrhea, epicutaneous sensitization, food allergy, intestinal pathology, oral tolerance

Introduction

Today, little is known of the underlying immunological mechanisms of food allergies. Progress in this field has also been hampered by lack of clinically relevant animal models. In the food allergy animal models described so far, sensitization routes have usually been either intragastric [1,2] or through intraperitoneal injections of the allergen along with immunological adjuvants such as Freund's complete adjuvant [3] or aluminum [4,5]. In these models, evidence to show gut pathology has remained vague.

Mast cells act at the earliest stages of an immune response, before the development of adaptive im-

mune responses that are necessary for B-cell isotype switching and subsequent F_C receptor-mediated activation [6]. Mast cells are found in connective tissue, being most abundant in skin, lung, and the gastrointestinal (GI) tract and along blood and lymphatic vessels. Two main types of mast cells have been described; tryptase-releasing connective tissue mast cells (CTMCs), and the tryptase and chymase-releasing mucosal mast cells (MMCs). The functions of mast cell proteases are largely unknown. They seem to be species related, the mast cell subtypes being based on their anatomical locations, and their differentiation is regulated by environmental stimuli [7]. In the mouse, MMCs are predominantly located within

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the mucosal epithelia [8]. Mucosal mast cell protease-1 (MMCP-1) is one of the 13 currently recognized mast cell-derived proteases in the mouse [9]. In murine parasite models, MMCP-1 has been detected in high concentrations in the serum, and in *Trichinella spiralis*-infected Balb/c mice, MMCP-1 has been shown to be a tight junction-breaking protease, thus, altering the permeability of the small intestine [10].

The aim of this study was to develop a food allergy model that did not rely on any adjuvants to artificially stimulate an immune response.

Material and methods

Sensitization and challenging protocols

Seven-week-old female Balb/c OlaHsd mice were purchased from Harlan (Horst, The Netherlands), maintained under specific pathogen free conditions. The animals were bred and kept on ovalbumin-free diet.

The mice were epicutaneously sensitized with 100 µg ovalbumin (fraction V; Sigma Chemical Co., St. Louis, Mo., USA) in 100 µl saline or sham-sensitized with saline. The epicutaneous sensitization was done according to Lehto et al. [11], with minor modifications. The mice were anesthetized with 4% isoflurane (Baxter, Abbott Laboratories, North Chicago, Ill., USA) with a flow of 400 ml/min by using the Univentor 400 Anesthesia Unit (AgnTho's AB, Lidingö, Sweden). The back of the mouse was shaved with an electric razor and the skin area exposed was lightly abraded by taping it four times with a special injection tape (Tegaderm, 3M; Health Care, Borken, Germany). The design for the experimental epicutaneous sensitization and intragastric challenges is illustrated in Table I and Figure 1: on day 1 (d1) and day 4 (d4), ovalbumin or saline was placed on a patch of sterile gauze (1 × 1 cm) which was taped to the skin of the mouse's back. On d8 the tape was removed. On d18 and on d22, an identical patch was reapplied to the same skin site and removed on d25.

From d40, challenging intragastric doses of ovalbumin (50 mg/in 200 µl saline) or saline were given to both groups starting on d40 (Table I) with a feeding needle (AgnTho's AB) at 2–3 day intervals (Figure 1). After the last dose had been given on d51, each individual mouse was kept separated in an

empty box and clinical symptoms occurrence was monitored for up to 1 h.

Blood samples were taken from the *vena saphena* during the experiment. The last blood samples were taken from the abdominal aorta (Figure 1), and tissue samples were taken at the same time.

MMCP-1 and specific IgE, IgG₁ and IgG_{2A} measurements

MMCP-1 was measured by ELISA according to the manufacturer's instructions (Moredun Scientific Ltd., Midlothian, UK).

Other antibodies used in the study were purchased from Beckton Dickinson (San Diego, Calif., USA). For ovalbumin-specific IgE, microtiter plates were coated with rat anti-mouse IgE (clone R35-72, 2 µg/ml) and the serum samples were diluted 1:40 in 10 mM phosphate-buffered saline (PBS). For measuring ovalbumin-specific IgG₁ and IgG_{2A}, the plates were coated with ovalbumin (fraction V, Sigma, 2 µg/ml), and the serum samples were diluted 1:22000 for IgG₁ and 1:180 for IgG_{2A} in 10 mM PBS.

For the detection of specific IgG₁ and IgG_{2A}, commercially available biotin-labeled anti-mouse isotype-specific secondary antibody was used at a concentration of 2 µg/ml (A85-3 MoAb; BD Pharmingen, San Jose, Calif., USA). For ovalbumin-specific IgE detection, the secondary antibody was biotinylated according to the manufacturer's instructions (EZ-Link Sulpho-NHS-Biotinylation Kit; Pierce Chemical Co., Rockford, Ill., USA) and fractionated with desalting columns (D-Salt Dextran Plastic Desalting Columns; Pierce Chemical Co.). The biotinylation ratio was estimated according to the manufacturer's instructions with HABA/avidin solution.

Streptavidin–horseradish peroxidase (BD Pharmingen) and peroxidase substrate reagents (Kirkegaard & Perry Laboratories, Gaithersburg, Md., USA) were used for immunodetection and the results were expressed as absorbances at 405 nm wavelength.

Histopathology and immunohistochemistry

Antrum, duodenum, jejunum, ileum, colon, and distal lung parenchyma specimens were embedded

Table I. Experimental set-up for the epicutaneous sensitizations and intragastric challenge trials.

Epicutaneous sensitization	<i>n</i>	Intragastric challenge	Abbreviation	<i>n</i>
Saline 100 µl	12	Saline 200 µl	Sal-sal	6
Saline 100 µl		Ovalbumin 50 mg/200 µl	Sal-ova	6
Ovalbumin 100 µg/100 µl	11	Saline 200 µl	Ova-sal	6
Ovalbumin 100 µg/100 µl		Ovalbumin 50 mg/200 µl	Ova-ova	5

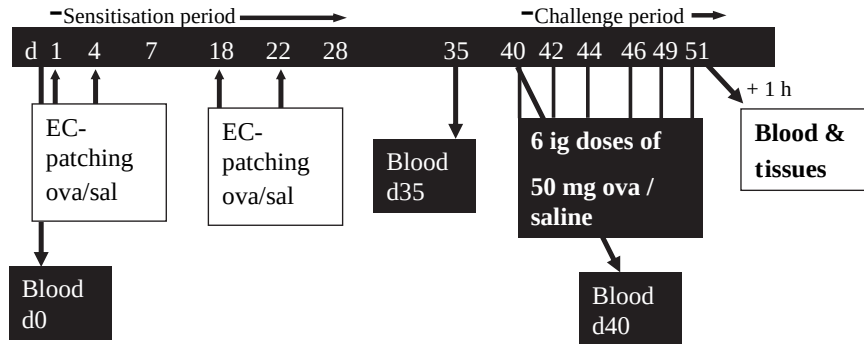


Figure 1. Design for the experimental sensitizations and challenges of the four trial groups. Thin lines starting from d40 indicate challenging doses. Abbreviations: EC = epicutaneous sensitization; d = day.

in 4% formaldehyde solution for hematoxylin-eosin (Merck, Darmstadt, Germany), o-toluidine blue (Fluka Chemie, Steinheim, Switzerland), or MMCP-1 monoclonal antibody (Moredun Scientific Ltd. Midlothian, UK) staining. Paraffin-embedded specimens were sectioned to 5 μ m. In addition, the sections for immunohistochemistry were fixed to super frost glasses at 58°C for 30 min.

For immunohistochemical staining, the samples were boiled twice in a microwave in 10 mM Tris/HCl–1 mM EDTA pH 9.0 and endogenous peroxidase activity was blocked with a 5% H_2O_2 solution. After washing and blocking with fetal calf serum, HRP-conjugated rabbit anti-mouse MMCP-1 primary antibody was incubated overnight at 4°C. A diaminobenzidine tetrachloride (DAB) SK-4100 peroxidase substrate kit was used for detection (Vector Laboratories, Burlingame, Calif., USA). Sections were then counterstained for 3 min with Mayer's hematoxylin.

Isolation of mRNA

Samples from the jejunum and ileum were homogenized in Trizol (Gibco BRL, Paisley, Scotland, UK) using an Ultra-Turrax T8 (IKA Labortechnik, Staufen, Germany). RNA extraction was performed according to the manufacturer's instructions. Briefly, contaminating genomic DNA was removed with DNaseI (RNase-free) (Gibco BRL) treatment, after which RNA was extracted with phenol/chloroform (1:1) (Gibco BRL) and reprecipitated, washed, and redissolved according to the Trizol instructions.

Synthesis, amplification, and quantification of reverse-transcribed complementary DNA (cDNA)

Transcribed cDNA was synthesized from 1 μ g of total RNA in a 50 μ l reaction mix using MultiScribeTM reverse transcriptase and random hexamers (PE Applied Biosystems, Foster City, Calif., USA). Polymerase chain reaction (PCR)

primers and probes were purchased from PE Applied Biosystems as predeveloped reagents. The real-time quantitative PCR was performed with the AbiPrism 7700 Sequence Detector System (SDS) (PE Applied Biosystems). For cDNA amplification, 10-min incubation at 95°C was done to activate AmpliTaqGold; this was followed by 40 cycles with 15 s at 95°C and 1 min at 60°C for each cycle. PCR amplification of the endogenous 18S rRNA was performed for each sample to allow normalization between samples. The results were expressed as relative units: i.e. the ratio of PCR amplification efficiency of sample cDNA to that of the calibrator (FAM C_T 40, VIC C_T 12.6 $\rightarrow \Delta C_T$ 27.4, where C_T denotes a threshold cycle).

Relative expression units (RUs) were calculated by the comparative C_T method. First, the C_T for the target amplicon (FAM) and the C_T for the endogenous control (VIC; 18S rRNA) were determined for each sample. The internal controls, ΔC_T , were subtracted from the ΔC_T of each experimental sample and termed as $\Delta\Delta C_T$. The amount of target normalized to an endogenous control and relative to the calibrator was calculated using the equation $2^{-\Delta\Delta C_T}$.

Statistics

Statistical analyses were performed with the SPSS11.5 program (SPSS Inc., Chicago, Ill., USA) using Student's *t*-test and the non-parametric Mann-Whitney test, as well as Schuirmann's two one-sided *t*-test procedures [12].

Results

Allergen exposure to lightly abraded skin initiated specific IgE production, which increased after oral challenge

After sensitizations on d35, there was an increase in average levels of ovalbumin-specific serum IgE

($p < 0.0001$) and IgG₁ ($p < 0.0004$) in ovalbumin-sensitized mice ($n = 11$) compared with sham-sensitized mice ($n = 12$) as analyzed by Wilcoxon's signed-rank test (Figure 2A, B, marked as d). During the challenge period there was a further significant increase in the mean levels of IgE and IgG₁ from d35 to d51 ($p < 0.003$, and $p < 0.004$, respectively, marked as e and f in Figure 2A, B). When the sal-ova group was compared with the ova-ova group, there was a significant difference in both IgE and IgG₁ on d51 ($p < 0.004$ and $p < 0.002$, respectively, analyzed with the Mann-Whitney test, and marked as g in Figure 2A, B).

Oral challenge of sham-sensitized groups did not induce any increase in serum immunoglobulin levels: average values in the sal-sal and sal-ova groups were similar with respect to both IgE and IgG₁ on d35, and, after the challenge period, on d51 (Figure 2A, B). Using the two one-sided t -test procedures of Schuirman [12], the difference between the groups with respect to IgE and IgG₁ was estimated to be at most 0.00841 and 0.00426 OD₄₀₅ units,

respectively, for d35, and 0.00526 and 0.0024 OD₄₀₅ units, respectively, for d51. As these upper limits can be regarded as negligible, the effect of oral ovalbumin exposure did not differ notably from that of saline, as measured by IgE and IgG₁.

There was no statistical difference in ovalbumin-specific IgG_{2A} levels between the study groups on d35 or d51, (data not shown).

Conventional histology, immunohistochemistry, and development of diarrhea, autopsy findings

Samples of antrum, duodenum, jejunum, ileum, colon, and lung were studied. In general, there was no consistent eosinophilia in ovalbumin-sensitized groups in hematoxylin-eosin staining (Figure 3A, B), and we could not find mast cells by toluidine blue staining (data not shown). Since murine intestinal MMCs stain poorly, if at all, with toluidine blue [13], we tried immunological staining for one of the subgroups of MMCs. For antigen, we chose MMCP-1, which in Balb/c mice had already been shown to play a critical role in the expulsion of parasites [14]. Only a few DAB-stained colored cells could be found in the control groups, whereas several such cells were seen in the ova-ova group, (Figure 3C, D). An increasing concentration gradient of MMCP-1⁺ cells from the duodenum to ileum was observed, with the highest values of MMCP-1⁺ cells in the duodenum and jejunum in the ova-ova group. We did not detect MMCP-1⁺ cells in the colon or lungs (Figure 4). The concentration gradients of the sal-sal and the ova-sal groups were similar to those of the sal-ova group (data not shown).

Diarrhea was not observed within 1 h after the third intragastric dose. Within 2 h after the fifth intragastric dose, 2/6 mice of the ova-ova group had diarrhea. Within 1 h after the last, sixth intragastric dose, we could clearly see watery diarrhea in 5/6 mice of the ova-ova group but in none of the control group individuals. At autopsy, in 4/6 of the animals in the ova-ova group, ceca or the areas from the ileum to the cecum were milky in color and the intestine content was of fluid consistency in comparison with the solid contents in the control groups (data not shown).

MMCP-1 appears in serum after intragastric challenges

There was no detectable MMCP-1 on d0 and d35 serum samples of the ovalbumin or sham-sensitized mice. On d40, 18 min after the first intragastric dose, serum MMCP-1 was undetectable (< 50 ng/ml) in the control groups, but increased to 1217 ± 241 ng/ml in the ova-ova group (Figure 5A).

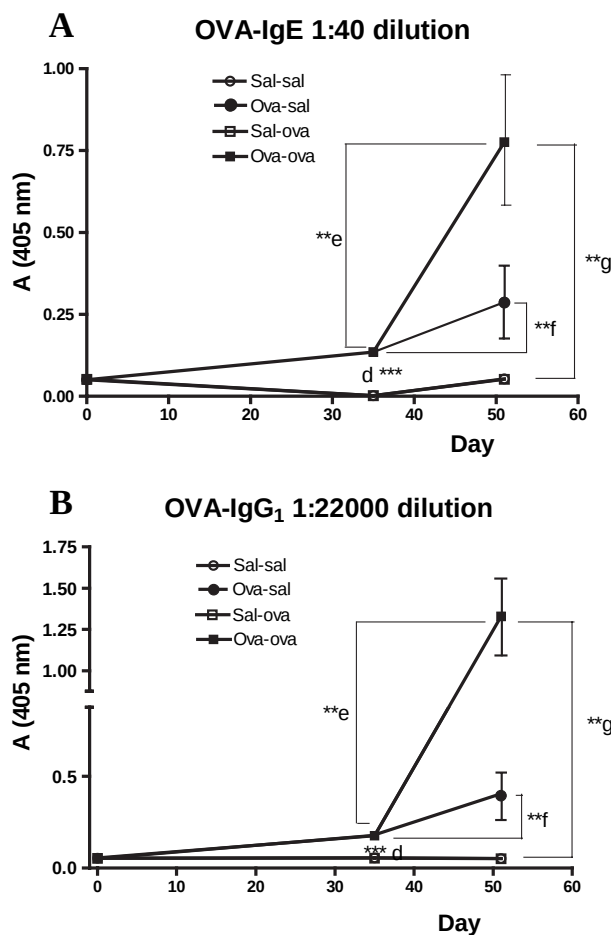


Figure 2. Serum ovalbumin-specific IgE (A) and IgG₁ (B). Standard deviations are shown in the figure, except those of d0 and d35, which are very small. For statistics and symbols of d, e, f, and g, see the text.

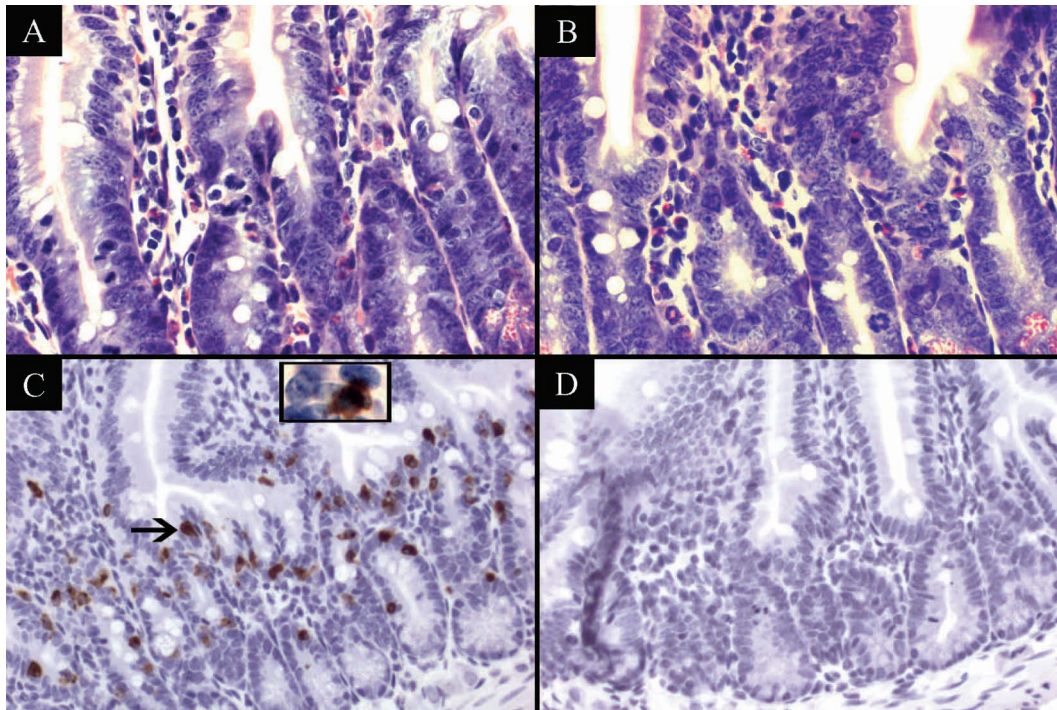


Figure 3. Hematoxylin-eosin staining from the jejunum of the ova-ova (A), and the sal-ova group (B), (magnification $\times 400$). Immunohistochemical staining of the MMCP-1⁺ cells, the ova-ova (C) and the sal-ova groups (D), (magnification $\times 200$). In (C) the arrow indicates an MMCP-1⁺ cell, the small figure shown at a magnification of $\times 400$.

Repeated intragastric challenges (6 times 50 mg ovalbumin) led to a massive increase in serum MMCP-1 concentration, on average $21,031 \pm 18,190$ ng/ml in the ova-ova group, whereas MMCP-1 was undetectable in the control groups (Figure 5B).

Real-time PCR showed decreased expression of Th-1-type response-related cytokines in the ileum

In ileum samples of the ova-ova group when compared with those of the control groups, there was a decrease in mRNA expression level of IFN- γ , a major cytokine of the Th-1-type response ($p < 0.05$). Furthermore, the mRNA expression level of CXCL-9 ($p < 0.05$), a Th-1 chemokine related to IFN- γ , was decreased and there was a trend towards a decrease in CXCL-10, but this was not significant. There was no significant difference in the mRNA expression of these cytokines in the jejunum samples (Figure 6).

IL-4 showed an increase in the jejunum samples of 3/5 allergic mice in the ova-ova but not in the control groups (Figure 6D), suggesting Th-2-directed skewing.

With the exception of IL-4, all of the studied cytokines, TGF- $\beta 1$, IL12/p35, IL-12/p40, IFN- γ , CXCL-9, and CXCL-10, showed lower expression levels in the jejunum than in the ileum, independently of the sensitization group (Table II).

Discussion

A new model of food allergy is described in which mice, sensitized epicutaneously with a protein antigen followed by multiple intragastric applications with the same antigen developed diarrhea and an accumulation of mast cells in the small intestine. At the time of appearance of clinical symptoms, vast amounts of MMCP-1 are released from these cells

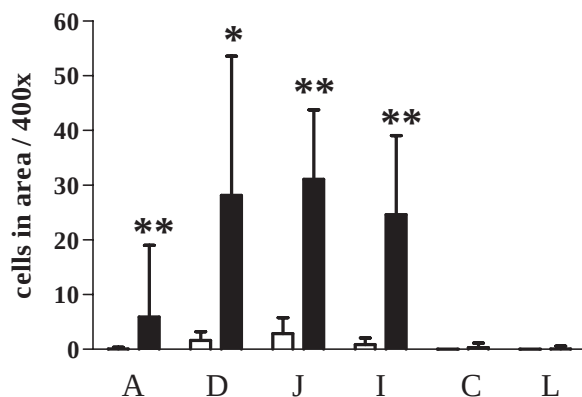


Figure 4. Distribution of the MMCP-1⁺ cells in different areas of the gastrointestinal (GI) tract. The ova-ova group is depicted as black bars and the sal-ova as white bars. The frequency of the MMCP-1⁺ cells was significantly higher in the ova-ova group than that in the sal-ova group; the statistical comparisons marked in the figure were calculated with the Mann-Whitney test: * $p < 0.05$; ** $p < 0.01$. Abbreviations: A = antrum; D = duodenum; J = jejunum; I = ileum; C = colon; L = distal lung.

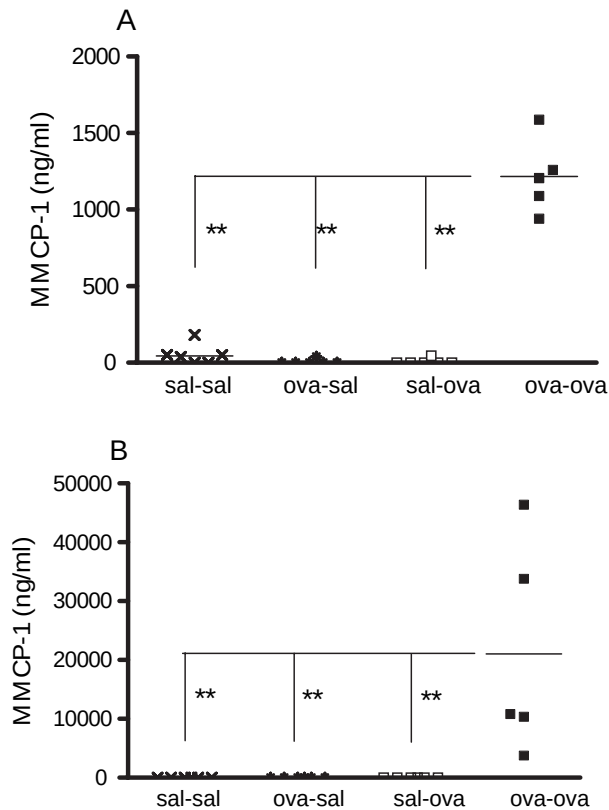


Figure 5. Serum MMCP-1 levels after intragastric doses. Eighteen minutes after the first intragastric dose on d40 (A) and 1 h after the last intragastric dose at sacrifice on d51 (B), the mean MMCP-1 concentrations were higher in the ova-ova group than those in any of the other study groups; statistical comparisons marked in the figure were calculated with the Mann-Whitney test: * $p < 0.05$; ** $p < 0.01$.

into the circulation. This procedure appears to represent a physiologically feasible model which mimics clinical human food allergy reasonably well. Importantly, immune responses were not artificially stimulated by any immunological adjuvants. However, our immunization protocol led to both functional changes (diarrhea and macroscopic autopsy findings) and mast cell-associated pathological alterations in the intestines of the animals. We have recently shown [15] that using a similar intracutaneous sensitization scheme followed by repeated antigen challenges to the respiratory tract, airway hyper-responsiveness to metacholine could be produced. This suggests that dermal sensitization routes can lead to apparent systemic allergy, denoted by vigorous IgE synthesis and Th-2 dominance in the affected skin, which can be directed by appropriate challenges to target organs elsewhere in the body.

Most of the previously described murine food allergy models [2,5,16,17] have suffered a lack of clear-cut immune-mediated hypersensitivity reactions in the intestinal tract. In most cases, the

histological findings have not been reported [1,16–19]. With regard to general intestinal pathology, non-specific pathological changes, such as shortened and thickened villi in the small intestine, have been observed in rat [5,20,21]. Slight sloughing of enterocytes at the tips of the villi in a mouse food allergy model has been reported [2], but in *N. brasiliensis*-immunized rats characterized by severe mastocytosis, a more clear-cut sloughing of villi was shown [21]. Increased amounts of red blood cells in the intestinal tract or in the lungs have been detected in mouse food allergy models [16,22,23]. In the murine models using immunostimulating adjuvants [3,4,23], there appears to be some evidence of local allergic inflammation detectable with conventional histological staining, e.g. accumulation of clustered lymphocytes, eosinophils, or mast cells.

Hsieh et al. [22] recently described an epicutaneous sensitization-based model of food allergy using a longer sensitization period than that used in the present study. Moreover, the model differed from the present study in the study design since they used only a single intragastric challenge of ovalbumin, and took blood and tissue samples relatively late, i.e. 6 h after the challenge. These investigators did not use immunostimulating adjuvants in their model and, they did not detect inflammatory cellular infiltrates in the intestines, although such cells were seen in the lungs. They noticed increased levels of plasma histamine, in addition to ovalbumin-specific IgE and IgG₁ but did not report diarrhea. Likewise, Brandt et al. [4], whose protocol included the non-physiological use of aluminum, did not observe diarrhea after the first or second 50 mg dose of ovalbumin, yet, diarrhea occurred in over 60% of the mice after the third challenging dose and in all mice after 6 to 10 challenging doses of ovalbumin.

Ovalbumin-specific IgE and IgG₁ increased in ovalbumin-sensitized animals before any intragastric challenges, which can be considered as an indication of successful induction of type 1 allergic response. We also noticed increases in specific IgE and IgG₁ in the ova-sal group between d35 and d51, which could originate from the relatively long exposure protocol; however, much higher increases were seen in the ova-ova group shortly after the intragastric doses. These results suggest that without dermal sensitization, followed by oral ovalbumin dosage cannot breakdown oral tolerance to ovalbumin, whereas oral challenges following dermal sensitization can markedly increase or potentiate specific antibody production. Obviously, in this model the production of IgE and IgG₁ is compatible with the build-up of Th-2 dominance, while IgG_{2A} plays an important role in the direction towards a Th-1-type response.

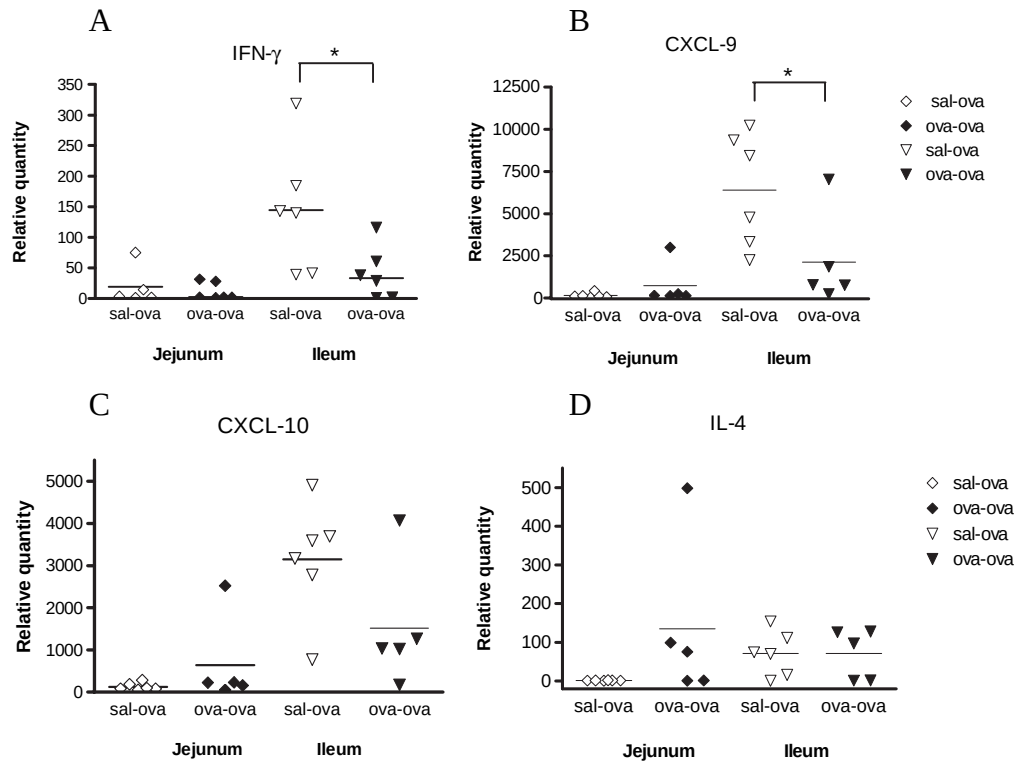


Figure 6. Real-time polymerase chain reaction (PCR) studies of cytokine mRNA expression from jejunum and ileum samples of the sal-ova and the ova-ova groups. Results of IFN- γ (A), CXCL-9 (B), CXCL-10 (C), and IL-4 (D) expression levels are shown. The horizontal lines denote medians of the groups, analyzed with the Mann-Whitney test: * $p < 0.05$; ** $p < 0.01$.

The present data suggest that serum MMCP-1, apart from being a marker of mucosal mastocytosis and of increased gut permeability [10], could be a new systemic serum marker of intestinal mast cell activation also in murine food allergy models. A marked increase in serum MMCP-1 was also noticed recently in aluminum sulfate-stimulated, intraperitoneally sensitized Balb/c mice after oral allergen challenge [4]. In the present study no MMCP-1 staining was detected in the colon or the distal lung tissue, which is not surprising as MMCP-1 seemingly is expressed only in a certain subtype of mast cells.

Our present results from the jejunum and ileum, and the preliminary results from the duodenum

showed low expression levels of IFN- γ mRNA in the duodenum. Since both intestinal microbial diversity and bacterial amounts gradually increase from the proximal to the distal end of the small intestine one might assume that the cytokine levels expressed are influenced accordingly. With regard to the studied mRNA expression patterns of the cytokines and chemokines in the GI-tract samples, mRNA expressions of IFN- γ and its related chemokine, CXCL-9, were decreased in the ova-ova group. There was a trend towards a decrease in CXCL-10 and an increase in IL-4, but these trends were not significant when compared to controls. The significantly decreased expression of Th-1 cytokines in the ileum indicates local development of Th-2 dominance.

Table II. Cytokine mRNA levels from the jejunum and ileum samples in relative expression units when compared with each individual 18 S rRNA level.

GI-area	TGF- β -1		IL-1 β		IL-12/p35	
	Average	st.dev.	Average	st.dev.	Average	st.dev.
Sal-ova jejunum	1265	1388	13	16	64	140
Ova-ova jejunum	1983	2983	15	25	111	229
Sal-ova ileum	6793	3584	182	78	300	146
Ova-ova ileum	5747	4638	200	201	120	120

The results were quantified by real-time polymerase chain reaction (PCR). In this analysis the results were calculated irrespective of the study groups.

Children with multiple food allergies were reported to have low expression of duodenal TGF- β 1 mRNA expression when compared with controls [24]. There was no significant difference in our results in the expression of TGF- β 1 in jejunum or ileum samples. In the production of MMCP-1 from bone marrow mast cell cultures, TGF- β 1 is needed together with stem cell factor, as well as IL-3, and IL-9 [25]. Thus, as there were high levels of MMCP-1 produced in the allergic mice in the present study, increased expression of TGF- β 1 mRNA could also have been expected.

The ovalbumin-sensitized and challenged mice developed diarrhea at the end of the experiment; i.e. after the sixth intragastric dose. This obviously reflects a pathological event in the GI tract but we could not record *in vivo* symptom scores from 1–5, as presented in the cholera toxin model [2] and in the model developed by Hsieh et al. [22]. We could see altered behavior in the ova-ova group of mice 0–60 min after the last challenges, mostly reflected as a tendency to stand still with stooped back. It is possible that the symptom scores referred to above come up differently in different animal models or in different murine strains (C3H/HeJ mice in the cholera toxin model), but the occurrence of visible diarrhea in the present model is obviously direct evidence of the course of pathologic events in the GI tract.

In conclusion, the present results show that specific allergic responses to a protein antigen can be targeted to the GI tract in epicutaneously sensitized mice. For the development of diarrhea, several repeated challenging doses are needed, while intragastric applications alone, without preceding skin sensitization, do not lead to manifestations of intestinal allergy. There is an influx of MMCs in the intestine and the activated cells release large amounts of MMCP-1 to the serum. We suggest that MMCP-1 could be used as a soluble marker for mast cell-dependent intestinal inflammation in murine models. The possibility of using mast cell proteases as markers of the allergic or inflammatory status of human small intestine should be further evaluated.

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