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Acute Allergic Skin Reactions and Intestinal Contractility Changes in Mice Orally Sensitized against Casein or Whey

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Key Words
Casein · Colon · Cow's milk allergy · Food allergy · IgE · Mast cell · Motility · Mouse model · Whey

Abstract
Background: Cow's milk allergy (CMA) is characterized by hypersensitivity against casein or whey, affecting 2.5% of young infants. The pathogenesis of CMA involves IgE as well as non-IgE-mediated reactions and clinical symptoms are found in the skin, lungs and gastrointestinal tract. In this study, local and systemic immunopathology was determined in whey- or casein-allergic mice. ***Methods:*** Mice were orally sensitized with casein or whey using cholera toxin as an adjuvant. Serum immunoglobulins and the acute allergic skin reaction (ear swelling 1 h after intradermal allergen challenge) were determined to reveal systemic hypersensitivity. Furthermore, pathophysiological changes were assessed within the intestine. ***Results:*** An acute allergic skin reaction was induced in both whey- and casein-sensitized mice. In these mice, whey-specific IgE, IgG_1, IgG_{2a} and casein-specific IgG_1 levels were found to be increased. In addition, the serum mouse mast cell protease-1 (mMCP-1) concentration was enhanced, reflecting mast cell degranulation. Indeed, the number of mMCP-1-positive mast cells within the colon was diminished in both whey- and casein-sensitized mice. Only in casein-sensitized mice isometric contraction of the colon was reduced, reflecting motility alterations. ***Conclusion:*** Mice, orally sensitized against casein or whey, revealed an allergen-specific acute allergic skin reaction. In casein-sensitized mice, hypocontractility of the colon reflected pathophysiological changes within the intestine. Allergen-induced ear swelling and intestinal contractility changes are novel parameters in animal models of CMA which may add to the search for new therapeutic strategies to relieve symptoms of CMA.

Introduction

Cow's milk allergy (CMA) is one of the leading causes of food allergy in adults [1]. In developed countries, approximately 2–3% of infants exhibit CMA. Although most infants outgrow CMA before their fifth year, IgE-mediated CMA predisposes the development of other (food) allergies and even asthma [2, 3]. Clinical features due to IgE-mediated reactions are expressed as immediate symptoms mostly. Clinical symptoms may involve the skin, respiratory tract and gastrointestinal tract, and can even lead to a systemic anaphylactic reaction [4, 5]. However, it should be realized that a particular group of patients, up to 40%, exhibit clinical features of CMA without detectable cow's milk-specific serum IgE [6–8]. So far,

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the only therapeutic approach has been the elimination of cow's milk proteins from the diet. Cow's milk contains 2 main protein classes, caseins (30 g/l) and whey proteins (5 g/l). The caseins consist mainly of αS1-, αS2- and β-casein, whereas whey proteins comprise of β-lactoglobulin, α-lactalbumin, bovine serum albumin, serum immunoglobulins and lactoferrin. Large population studies with cow's milk-allergic infants have shown that the major allergens are β-lactoglobulin, caseins and α-lactalbumin [9, 10]. The pathogenesis and development of CMA probably involves a partial dysfunction in immunological tolerance induction during early life combined with enhanced intestinal permeability [5]. There is growing evidence that intestinal allergic responses can initiate motility changes through attraction and activation of mast cells and production of Th2 type of cytokines [11]. Motility changes of the intestine are a clinical problem in CMA patients, which manifests as either diarrhea or even constipation [12–14].

Animal models of CMA provide a tool to reveal mechanisms involved in CMA and may explore new therapeutic and preventive approaches in those models. However, in most existing food allergy models, the animals are not sensitized via the oral route (but, for example, intraperitoneally), while in reality humans are sensitized orally. Although numerous animal models for food allergy are available using an intraperitoneal sensitization protocol, only a few models use oral sensitization [15–23]. Li et al. [22] were the first to introduce a model in which mice were sensitized orally against complete cow's milk, while Frossart et al. [23] showed oral sensitization to β-lactoglobulin. In these models, mice are sensitized by means of intragastric (i.g.) gavage using cholera toxin (CT) as an adjuvant. The primary read out parameters are IgA and IgE, and in particular cow's milk-specific IgE. Furthermore, Li et al. [22] described systemic anaphylaxis upon i.g. challenge with cow's milk. They described vascular leakage, mast cell degranulation and enhanced serum histamine levels in CMA animals. The systemic allergic responses to food allergens that are described in these models resemble features found in the clinic; however, the mechanisms behind the development of CMA are still only partially understood.

To further assess mechanisms underlying CMA and/or test new concepts for prevention and/or treatment for CMA, in the present study new tools to address the pathological changes in casein and whey allergy in mice have been developed. Measurement of the acute allergic skin response, as an equivalent of the human skin prick test, was introduced to reflect a systemic sensitization. Intestinal contractility changes were measured to address local pathophysiological changes after oral challenge with the specific allergens.

Methods

Chemicals

Casein and whey were obtained from DMV International (Veghel, The Netherlands). Cholera toxin was purchased from Quadratech Diagnostics (Epsom, UK) and PBS from Cambrex Bio Science (Verviers, Belgium). Biotin-labeled rat anti-mouse IgE, IgG_1 and IgG_{2a}, and unlabeled rat anti mouse IgE were obtained from BD Biosciences (Alphen aan den Rijn, The Netherlands). All other chemicals were obtained from Sigma-Aldrich-Chemie (Zwijndrecht, The Netherlands).

Oral Sensitization and Challenge of Mice

Three- to five-week-old specific pathogen-free female C3H/HeOuJ mice (4–6 mice per group) were purchased from Charles River Laboratories (Maastricht, The Netherlands), maintained on cow's milk protein-free mouse chow (Special Diets Services, Witham, UK) and housed in the animal facility at the Utrecht University. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Mice were sensitized i.g. with 0.5 ml homogenized casein or whey (40 mg/ml PBS) with CT (20 μg/ml PBS) as an adjuvant, using a blunt needle. Control mice received CT alone or PBS. Mice were boosted weekly for a period of 4–6 weeks, 1 week after the last sensitization mice were challenged i.g. with 100 mg casein or whey in 1 ml PBS (figure 1). Blood samples were collected and centrifuged (15 min at 13,500 rpm). Sera were stored at –70°C. Mice were sacrificed by cervical dislocation 30 min after i.g. challenge.

Allergen-Specific Skin Response

The acute allergen-specific skin response was measured after injection of the specific protein in the ear pinnae intradermally (i.d.). Before i.g. challenge (t = 0), the control, casein- and whey-sensitized mice were injected i.d. in the left ear with 20 μl homogenized casein or whey (0.5 mg/ml in PBS), respectively. In the right ear, 20 μl PBS was injected as a vehicle control. Also, the CT and PBS sham-sensitized mice received a casein or whey ear challenge using PBS injections as control. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands), at t = 0 as well as 1, 4 and 24 h after challenge. The allergen-specific net ear swelling was calculated by subtracting the basal thickness (0 h) from the thickness measured at 1, 4 and 24 h after injection in the left ear. In addition, the control (right ear) swelling measured at the same time points was subtracted. The ear swelling is expressed as delta μm.

Measurement of Serum Immunoglobulin and Mouse Mast Cell Protease-1

Concentrations of total IgE and levels of casein- or whey-specific IgE, IgG_1 and IgG_{2a} were determined in serum of sacrificed mice by means of ELISA. Microlon plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 100 μl whey or casein (20 μg/ml) in coating buffer or rat anti-mouse IgE (1 μg/ml;

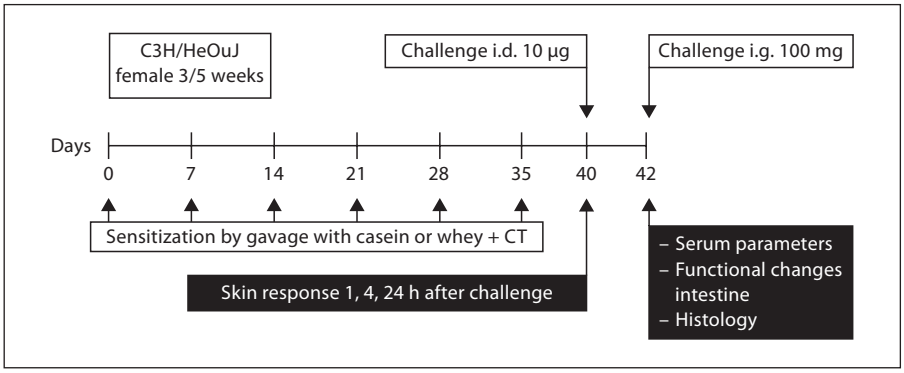


Fig. 1. A schematic overview of the sensitization and challenge protocol and the parameters that are analyzed.

carbonate-bicarbonate buffer, 0.05 M, pH = 9.6) for 18 h at 4°C. Plates were washed and blocked for 1 h with 5% BSA. Serum samples were applied in several dilutions (4–100 times) and incubated for 2 h at room temperature. Plates were washed and incubated with biotin-labeled rat anti-mouse IgE, IgG_1 or IgG_{2a} (1 µg/ml) for 90 min at room temperature and washed. The plates were incubated with streptavidin-horseradish peroxidase for 1 h, washed and developed with *o*-phenylendiamine. After 5 min, the reaction was stopped with 4 M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Bio-Rad, Hercules, Calif., USA). Serum concentrations of mouse mast cell protease-1 (mMCP-1) were determined as described previously using a commercially available ELISA kit (Moredun Scientific Ltd., Penicuik, UK) [24].

Immunohistology of the Colon

For determination of histopathological alterations, Swiss rolls of 4 cm colon were prepared [25]. The colon was carefully dissected, opened longitudinally over the mesenteric line and luminal contents were removed by gently washing in saline. The colon was placed with the mucosal side down. Colons were rolled from the distal to the proximal end, fixed in ice-cold 10% formaldehyde in PBS (for 24–48 h) and embedded in paraffin (Leica EG1150c; Leica Microsystems, Rijswijk, The Netherlands). Sections of 5 µm were cut using a microtome (Leica RM2165; Leica Microsystems), stretched on water (Tissue Flotation Bath TFB45; Medite, Nunningen, Switzerland) and placed on poly-L-lysine-coated slides. Deparaffinized Swiss roll sections were stained with mMCP-1 to detect mucosal mast cells [26]. In short, after fixation in acetone, the sections were blocked with 10% normal goat serum, followed by incubation with rat anti-mouse mMCP-1 (kindly donated by Dr. H.R.P. Miller, Edinburgh, UK). The primary antibody was detected with a biotinylated polyclonal goat anti-rat antibody (Pharmingen, Aalst, Belgium), followed by incubation with streptavidin-horseradish peroxidase (Sanquin, Utrecht, The Netherlands). Color was developed with AEC chromogen staining kit (Sigma Chemical Co., St. Louis, Mo., USA) and sections were counterstained with hematoxylin. Mast cell counts were performed by counting 1 complete section of every animal.

Isometric Contraction of the Colon

The colon, caudal from the cecum, was dissected free of connective tissue and mesenterium. Colon parts of 1 cm length were mounted in an organ bath, using 2 small clamps, containing 10 ml tyrode buffer (in mM: NaCl 136.89, KCl 2.68, $MgCl_2$ 1.05, $CaCl_2$ 1.77, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 5.55) [27–29]. The tissue was kept at 32°C, to prevent spontaneous contraction, and continuously gassed with a 5% CO_2 and 95% O_2 gas mixture. One clamp was attached to a fixed point in the organ bath and the other clamp was connected to an isometric transducer (Harvard Apparatus Ltd., Edenbridge, UK) with an analog recorder (BD40; Kipp & Zn., Delft, The Netherlands). Contractions were measured under a constant preload of 1.00 g. The preparations were equilibrated for 1 h in the organ bath before starting a log dose-response curve for carbachol (10^{-8} until 10^{-3} M). After every dosage, the organ bath was flushed twice and the tissue was allowed to recover for 10 min before addition of the next concentration. Also, basal activity and spontaneous contraction frequencies of the tissue were recorded.

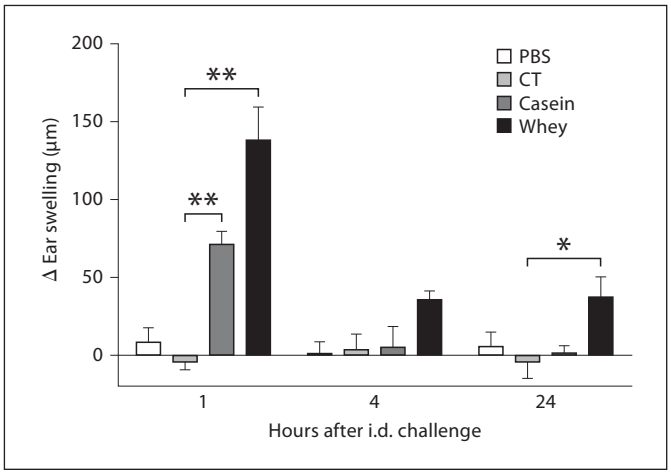


Fig. 2. Induction of an acute ear swelling in casein- and whey-sensitized mice in comparison with sham-sensitized (CT and PBS) mice. Δ ear swelling is calculated by subtracting the specific ear swelling induced by the corresponding antigen with vehicle (PBS)-induced swelling at 1, 4 and 24 h. * $p < 0.05$; ** $p < 0.01$; n = 4 in PBS group and n = 6 in all other groups.

Water Percentage in the Stool

Twenty-four hours after oral challenge, feces were collected from every animal. These samples were weighted, dried for several days and weighted again. The difference in weight is the evaporated water. The relative amount of water in the feces (relative to the total weight of the feces) was calculated and statistically evaluated.

Statistics

All data except for the isometric contractions were analyzed using one-way ANOVA and post hoc Dunnett's test. Isometric contraction data were analyzed using repeated measures ANOVA and post hoc Dunnett's test. Statistical analyses were conducted using GraphPad Prism software (version 4.0). For correlations, the Spearman rank order correlation coefficient test was used in SPSS 13.0 for Windows. Data are represented as means ± SEM.

Results

Acute Allergen-Specific Skin Reaction

In order to study allergic skin responses, mice were challenged i.d. in the ear pinnae with casein or whey 1 week after the last oral sensitization. One hour after dermal challenge, the allergen-specific acute ear swelling response was maximal when compared to PBS and CT sham-sensitized control mice. The delta ear swelling in the casein- and whey-sensitized animals was 71.2 ± 8.4 and 137.9 ± 21.7 μm, respectively, while this was neglectable in PBS and CT sham-treated mice (8.1 ± 9.4 and –4.6 ± 4.7 μm, respectively; fig. 2, $p < 0.01$, n = 4 in PBS group, n = 6 in all other groups). All sensitized mice reacted positively to the allergen, indicating that there are no nonresponders. Four hours after challenge, the ear swelling turned to basal levels in the casein group, while in the whey-sensitized group the swelling remained slightly enhanced up to 24 h after swelling (37.5 ± 12.6 μm in whey-sensitized vs. –3.9 ± 11.4 μm in CT-sensitized animals, $p < 0.05$). In additional experiments (data not shown) it was found that the marginal 24 h response was not followed by a response at later time points. In sham-sensitized animals, no differences were found between casein and whey challenge at any time points. Furthermore, whey challenge in casein-sensitized mice or vice versa did not result in any significant swelling response, indicating the antigen specificity of the skin reaction (data not shown).

Increased Total IgE and Antigen-Specific Serum IgE, IgG_1 and IgG_{2a} Levels

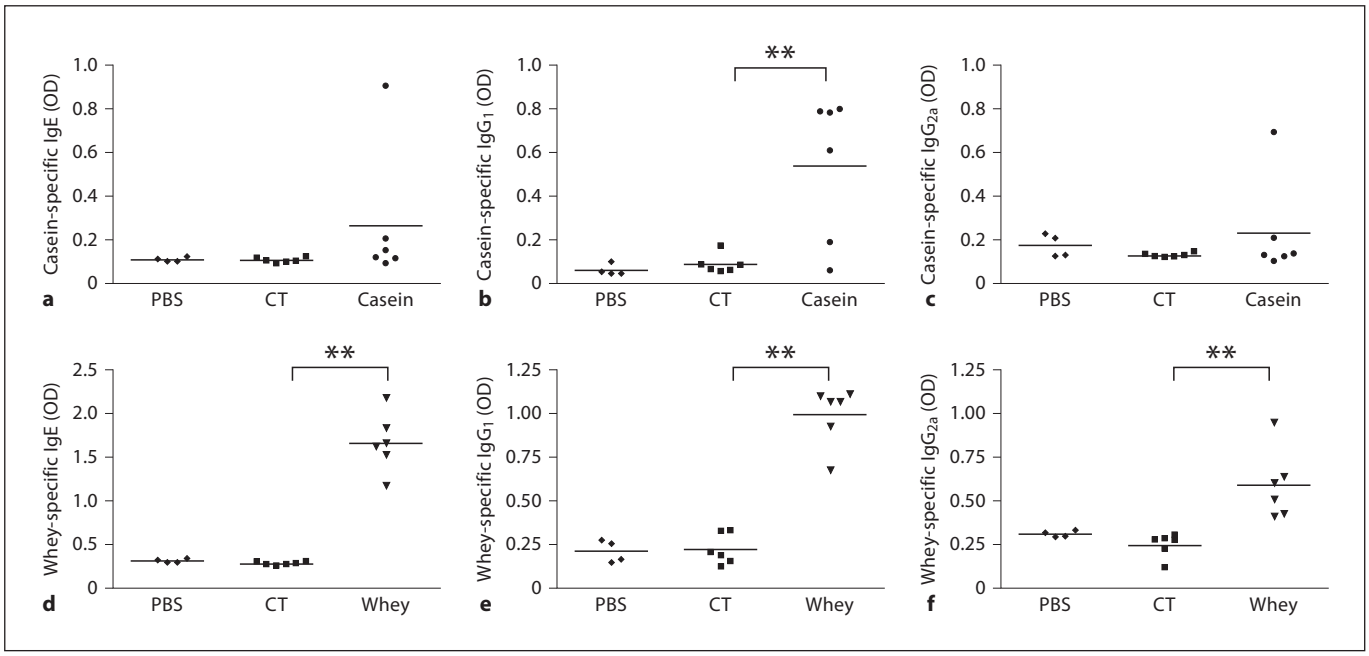
Half an hour after oral challenge, serum was obtained from both sensitized and nonsensitized animals. Total IgE concentrations were increased in casein- and whey-sensitized mice (400 ± 32 and 364 ± 19 ng/ml, respectively) in comparison with CT and PBS sham-sensitized mice (243 ± 37 and 218 ± 31 ng/ml, respectively; data not shown, $p < 0.01$ or $p < 0.05$, n = 4 in PBS group and n = 6 in all other groups). Casein-specific IgE as well as casein-specific IgG_{2a} levels were not increased (fig. 3a and c). In contrast, casein-specific IgG_1 levels were enhanced in the casein-sensitized mice (0.537 ± 0.135 OD_{A490}) when compared to CT and PBS sham-sensitized animals (0.087 ± 0.017 and 0.062 ± 0.013, respectively; fig. 3b, $p < 0.01$). In the whey-sensitized animals, the whey-specific IgE (1.662 ± 0.136), IgG_1 (0.988 ± 0.069) and IgG_{2a} (0.587 ± 0.081) levels were augmented when compared to the CT sham-sensitized controls (0.000–0.311 ± 0.032; fig. 3d–f, $p < 0.01$).

Correlation Serum Immunoglobulins and the Acute Allergic Skin Reaction

To investigate the correlation between serum immunoglobulins and the ear swelling response, linear regression analyses were performed using data from 4–5 independent experiments. The serum dilutions used to calculate the correlations were: 10 times for IgE, 100 times for IgG_1 and 50 times for IgG_{2a}. In whey-sensitized mice the acute ear swelling was found to correlate positively with whey-specific IgE (fig. 4c; $p = 0.015$, $r^2 = 0.276$, n = 21), whey-specific IgG_1 (fig. 4d; $p = 0.043$, $r^2 = 0.154$, n = 27), but not with whey-specific IgG_{2a} (data not shown; $p = 0.314$, $r^2 = 0.038$, n = 29). Ear swelling of casein-sensitized mice was not found to correlate with casein-specific IgE (fig. 4a; $p = 0.342$, $r^2 = 0.033$, n = 29), casein-specific IgG_1 (fig. 4b; $p = 0.521$, $r^2 = 0.015$, n = 29) and casein-specific IgG_{2a} (data not shown; $p = 0.488$, $r^2 = 0.18$, n = 29).

Fig. 3. Enhanced serum immunoglobulins in casein- and whey-sensitized mice. **a–c** In the casein-sensitized mice, only casein-specific IgG_1 levels were enhanced, no specific IgE and IgG_{2a} was measured. **d–f** In the whey-sensitized mice, specific IgE, IgG_1 and IgG_{2a} serum levels were enhanced. ** $p < 0.01$; n = 4 for PBS and n = 6 for all other groups.

Fig. 4. Regression analyses between ear swelling and immunoglobulin for casein- and whey-sensitized mice. Correlation between ear swelling and casein-specific IgE (**a**; $p = 0.342$, $r^2 = 0.033$, n = 29), casein-specific IgG_1 (**b**; $p = 0.521$, $r^2 = 0.015$, n = 29), whey-specific IgE (**c**; $p = 0.015$, $r^2 = 0.276$, n = 21) and whey-specific IgG_1 (**d**; $p = 0.043$, $r^2 = 0.154$, n = 27).


Casein-specific IgE (OD)
Casein-specific IgG1 (OD)
Casein-specific IgG2a (OD)
Whey-specific IgE (OD)
Whey-specific IgG1 (OD)
Whey-specific IgG2a (OD)
PBS
CT
Casein
Whey
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a
b
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e
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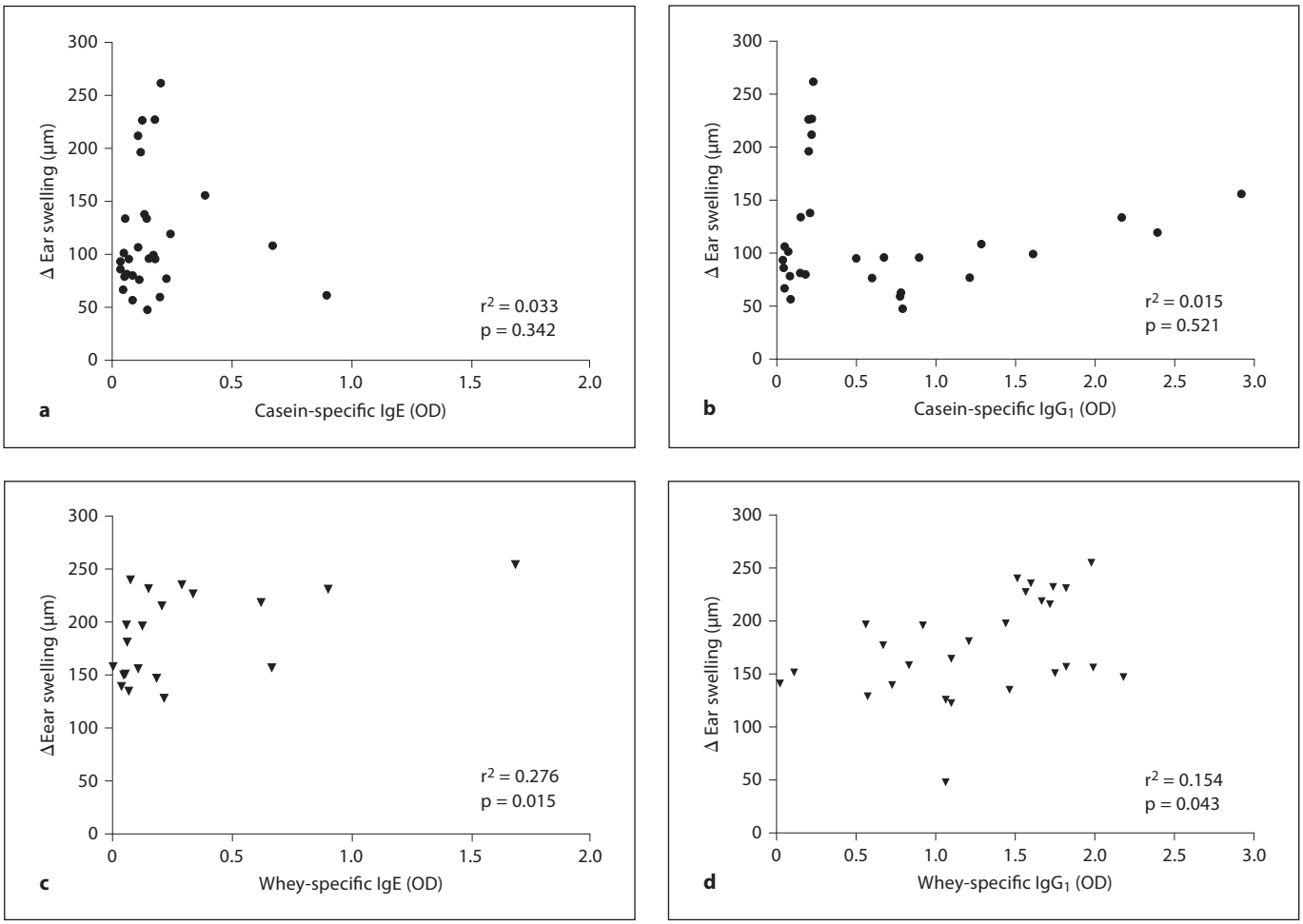
3


Δ Ear swelling (μm)
ΔEar swelling (μm)
Casein-specific IgE (OD)
Casein-specific IgG1 (OD)
Whey-specific IgE (OD)
Whey-specific IgG1 (OD)
r2 = 0.033
p = 0.342
r2 = 0.015
p = 0.521
r2 = 0.276
p = 0.015
r2 = 0.154
p = 0.043
a
b
c
d

4

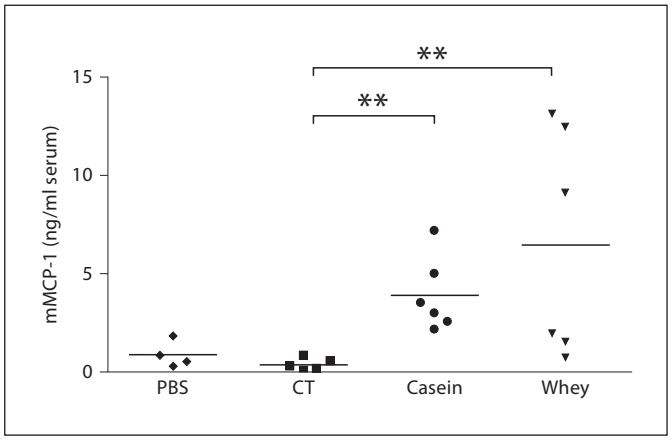


Fig. 5. Serum mMCP-1 concentrations are increased in casein- and whey-sensitized mice in comparison to sham-sensitized (CT and PBS) mice. ** $p < 0.01$; n = 4 for PBS, n = 5 for CT and n = 6 for all other groups.

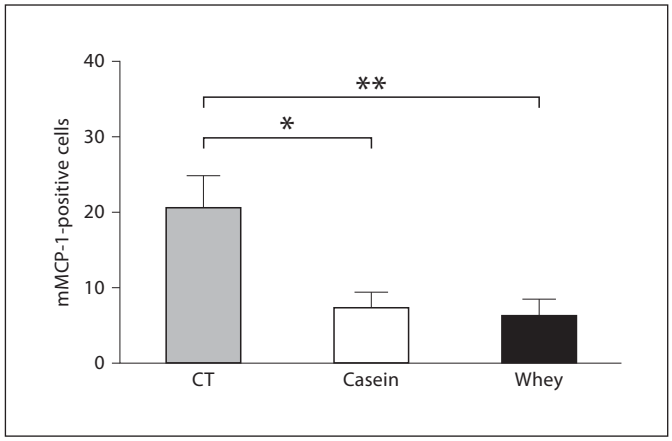


Fig. 6. Decreased mast cell counts in colon of casein- and whey-sensitized mice. Immunohistochemistry staining of mMCP-1 in 5-μm sections. The mean number of mMCP-1-positive cells was declined in casein and whey mice when compared to CT mice. * $p < 0.05$; ** $p < 0.01$; n = 6 for all groups.

Increased mMCP-1 Levels in Serum

mMCP-1 is a protease specific for mouse mucosal mast cells and will appear in the bloodstream after mast cell degranulation. To assess mast cell degranulation, mMCP-1 levels were determined in the serum 30 min after oral challenge. mMCP-1 serum concentrations of casein- and whey-sensitized mice were enhanced when compared to CT and PBS controls (3.890 ± 0.769 and 6.472 ± 2.341 vs. 0.357 ± 0.139 and 0.878 ± 0.338 ng/ml, respectively; fig. 5; n = 4–6, $p < 0.01$).

Decreased Number of mMCP-1-Positive Mast Cells in the Colon

After oral challenge, mice were sacrificed and the colon was obtained for histological examination. mMCP-1-positive cells were counted after immunohistochemical staining. A decreased number of mucosal mast cells was counted in the colon of casein- (7.33 ± 5.13; $p < 0.05$) and whey-sensitized animals (6.33 ± 5.32; $p < 0.01$) when compared to nonsensitized control mice (20.67 ± 10.27; fig. 6; n = 6).

Altered Isometric Contraction of the Colon

Isometric contraction of the colon was determined by assessing contractility upon exposure to muscarinic receptor agonist carbachol (noncumulative dose response, 10^{-8} – 10^{-3} M). Reduced contractility for all carbachol concentrations was found for the colon of casein-sensitized mice when compared to sham-treated mice (fig. 7; $p < 0.01$, basal contractility did not differ between groups). This hyporesponsiveness of the colon cannot be characterized by a right movement of the EC50 concentration, but only by a reduction in maximal contraction force (E_{max}) (4,975 ± 1,046, 2,242 ± 296 and 5,933 ± 1,071 mg in control, casein and whey groups). The hyporesponsiveness of the colon in the casein-sensitized mice was found to be consistently present in all experiments, while none of these effects were seen in the whey-sensitized animals.

Water Percentage in the Stool

The relative amount of water in the feces was increased in the casein- (fig. 8; 61.97 ± 0.97%, $p < 0.05$, n = 6) and whey-sensitized animals (63.22 ± 1.78%, $p < 0.01$, n = 6) when compared to the control mice (56.68 ± 0.62%, n = 6).

Discussion

One of the most common food allergies in childhood is CMA. CMA is diagnosed when symptomatic patients have enhanced serum levels of cow's milk protein-specific IgE (radioallergosorbent test) and/or a positive skin prick test (SPT). In addition, a double-blind placebo-controlled oral challenge can be performed, which is the most reliable test for food allergy [9, 30, 31]. Currently, no immunotherapy is available for CMA patients, hence pa-

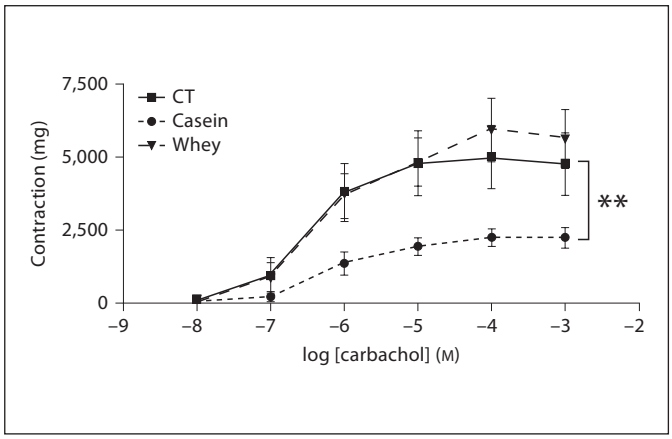


Fig. 7. Contractility changes in the colon of casein-sensitized mice after carbachol stimulation. Contractility of the colon of casein mice was decreased when compared to CT- and whey-treated mice. ** $p < 0.01$; n = 6 in all groups

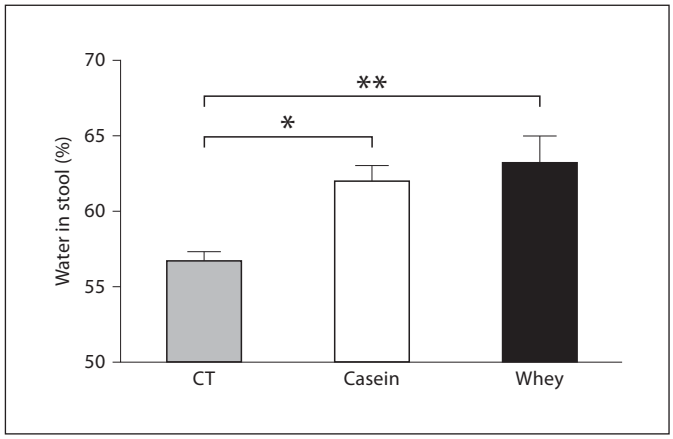


Fig. 8. Percentage of water in the stool of casein- and whey-sensitized mice compared to CT controls. The stool water content was found to be increased in both casein and whey mice, 24 h after oral challenge. * $p < 0.05$; ** $p < 0.01$; n = 6 in all groups.

tients need to avoid cow's milk allergens in the diet and use hydrolyzed milk formulae [9]. In the present study, sensitization against the cow's milk proteins whey and casein is investigated in mice that were sensitized orally. Specific immunoglobulin levels were induced and local and systemic symptoms were evaluated by studying effects in the gastrointestinal tract and skin. The investigated parameters closely resemble diagnostic tools that are used in the clinic; they are summarized in table 1.

Besides screening for cow's milk-specific serum IgE, the SPT is used for the diagnoses of CMA in humans [30]. In the current study, the allergen-induced ear swelling is introduced as a possible equivalent for the SPT. It is a new tool to determine systemic sensitization for casein and whey in mice. Oral sensitization with casein or whey consistently resulted in a positive acute allergic ear swelling response upon local allergen challenge. Furthermore, it can be concluded that sensitization via the oral route was able to induce systemic sensitization in mice treated either with casein or whey. In other models for skin hypersensitivity, comparative values for ear swelling responses have been found [32].

Although both whey- and casein-sensitized animals showed evidence of systemic allergy according to the ear swelling response, differences were found in serum immunoglobulin responses between the groups. Although total IgE concentrations and specific IgG_1 levels were enhanced in both the casein- and whey-sensitized mice, specific IgE and IgG_{2a} were found to be increased only in the whey-sensitized mice. In the clinic, 40% of CMA patients have a negative radioallergosorbent test for cow's milk proteins; however, they reveal to be positive in the SPT [6–8]. In a recent study, the SPT was found to correlate better with the double-blind food provocation test than serum immunoglobulins, although larger studies are necessary to confirm these findings [33]. Typically, in the casein-sensitized mice, no specific IgE was detected, while the mice indeed showed systemic allergy as seen in the acute allergen-induced ear swelling response. In contrast, the whey-sensitized mice showed the classical characteristics of type I IgE-mediated allergy, scoring positive for allergen-specific IgE and acute ear swelling, underlining the validity of this tool [34–36]. In whey-sensitized mice, the validity of the acute skin reaction test as a tool

Table 1. Overview of major parameters tested in both models

	Parameter	Casein	Whey
Sensitization	IgE	ND	+
	IgG_1	+	+
	ear swelling	+	+
Mast cell degranulation	mMCP-1	+	+
Physiology	water percentage	+	+
	motility	–	ND

ND = No difference observed compared to control (CT) mice; + = increased compared to control (CT) mice; – = decreased compared to control (CT) mice.

to determine systemic sensitization was supported by the finding that challenge-induced ear swelling correlated with whey-specific IgE and IgG_1. The acute allergic ear swelling in casein-sensitized mice was not associated with enhanced specific IgE levels, which may imply that there is no role for IgE in this model under the current protocol. In CMA patients, casein-specific IgE can be detected in the serum, but levels vary. It is known that patients with persistent CMA over the age of 9 years had elevated levels of casein-specific IgE compared to younger children with CMA. Therefore, low levels of casein-specific IgE early in life could indicate a nonpersistent form of CMA [31]. Instead of IgE, the acute ear swelling in mice might have been triggered by IgG_1, which is generally known to play a crucial role in mast cell activation in rodents. It has been shown that immunoglobulins can significantly interfere, positively and negatively, with mast cell responses (reviewed by Bruhns et al. [37]). In addition, it is known from the literature [38] that even in FcεRI-deficient mice, anaphylactic reactions are possible, indicating that IgE is not always a prerequisite for anaphylaxis. Although in casein-sensitized mice no significant correlation was found between ear swelling and levels of IgG_1, the scatter plot indeed shows a subgroup of mice in which the ear swelling is positively associated with the level of IgG_1. Hence, although casein-allergic mice lack specific IgE, enhanced IgG_1 levels in these mice may at least partly reflect allergic sensitization. Apart from IgE and IgG_1, Redegeld et al. [39] have found that mast cell degranulation can occur with immunoglobulin light chain. Immunoglobulin light chain is produced in excess during the formation of immunoglobulins. Hence, casein-specific immunoglobulin light chain may have caused the ear swelling in casein mice with an acute allergic skin response that could not be explained by the presence of IgE nor IgG_1. However, this remains speculative and further research is necessary.

Whey-sensitized mice show a slight but significant ear swelling at 24 h. Together with the higher allergen-specific IgG_{2a} levels in these animals, this might reflect a late-phase response after whey sensitization. Frossard et al. [40] also found enhanced serum levels of IgE/IgG_1 and IgG_{2a}, characteristic for a mouse Th_2 and Th_1 type of immune response, respectively, against β-lactoglobulin in a C3H/HeOuJ mice model for β-lactoglobulin. These levels of IgG_{2a} are in accordance with enhanced levels of IgG_{2a} that have been found in a similar mouse model for peanut allergy [22, 23, 41].

Clinical features of CMA are generally known to be elicited by mucosal mast cells [4, 22, 42]. Those mast cells are present in the intestinal mucosa and additionally drawn to the site of allergen challenge. In casein- and whey-sensitized mice, mast cell numbers of the colon were found to be declined in comparison to sham-sensitized mice. Mucosal mast cells were stained with mMCP-1, a β-chymase present in the mast cell granules, which end up in the bloodstream after degranulation [43]. In both whey- and casein-sensitized mice, serum mMCP-1 concentrations were increased, 30 min after i.g. challenge, reflecting mast cell degranulation. Upon oral allergen exposure, mucosal mast cells may have degranulated, resulting in enhanced mMCP-1 serum levels. After mast cell degranulation, the mast cell itself is not visible anymore with mMCP-1 staining, which might explain the drop in mast cell numbers in the colon of casein- and whey-sensitized mice [44].

One of the most prominent symptoms of food allergy is discomfort in the gastrointestinal tract which can be abdominal pain, diarrhea or sometimes constipation [4, 5]. The collected stool samples of casein- and whey-sensitized animals contained a higher water percentage than the controls, which is suggestive of diarrhea. Besides diarrhea, dysmotility of the intestine is a problem in CMA patients [12, 13]. The casein-sensitized mice showed contractility changes in organ bath studies, while whey-, CT- and PBS-sensitized mice did not. Isometric contractions of colon segments of casein-sensitized animals were hyporeactive in comparison to CT controls. It is known that allergy has adverse effects on the gut health, causing dysmotility [45, 46]. In this study, smooth muscle contractility differences between control and casein-sensitized animals may reflect changes in motility, which is supported by findings of Kobayashi et al. [47] in a model for diarrhea. Motility measurements are often performed when studying inflammatory bowel diseases, in which similar intestinal symptoms can be found. In these studies, local intestinal inflammation is indicated as causative factor for motility changes reflecting alterations in smooth muscle contractions [48]. Hence, local intestinal inflammation may have induced hypomotility occurring in the casein mice. However, histological evaluation did not reveal any obvious signs of inflammatory cell infiltrates after oral challenge. Possibly during sensitization, casein might have caused local inflammation, hereby reducing sensitivity of cholinergic neurons for carbachol stimulations, known to alter colonic motility [49]. In addition, local mast cell degranulation may have induced motility changes [11]. Mule et al. [29, 50] have shown that activation of protease-activated receptors (PAR) can cause colon smooth muscle relaxation as well as contraction. PAR

agonists such as mast cell-derived tryptase may have caused PAR activation in both casein- and whey-sensitized mice. It remains speculative why hypomotility of the colon was only observed in casein-sensitized mice; however, local levels of tryptase and PAR expression may differ between casein- and whey-sensitized mice and will be the focus of future investigation.

Additionally, casein and whey proteins differ with regard to physical, physiological and dietary properties and therefore may cause differential effects within the intestine. For example, it is known that there is a difference in digestive speed of casein and whey [51]. Casein protein is a slowly digested dietary protein, while whey protein is a fast one [52]. In addition, both proteins have different effects on satiety and gastrointestinal hormone response [53]. Hence, these proteins differentially influence the intestinal physiology. Furthermore, it is known that casein by itself can cause DNA damage in the colon, which is associated with a thinner mucus barrier. In the same study, whey did not cause these effects [54]. Two studies have shown hypomotility of the intestine after ingestion of casein, which might relate to the presence of casomorphins that were found to reduce motility [55, 56]. Hence, casein may also have intrinsic properties that can cause hypomotility. At this stage, the exact mechanism behind the hypocontractility found in the casein-sensitized mice is not known and will be the topic of future studies.

Taken together, altered motility suggests subtle local changes in intestinal discomfort and provides a new tool to measure local intestinal alterations as a consequence of allergic sensitization in mice.

Both casein and whey sensitization consistently resulted in an acute allergic skin reaction after allergen challenge, which was associated with specific IgE and IgG_1 serum levels in whey-sensitized mice and specific IgG_1 serum levels in casein-sensitized mice. Decreased numbers of mMCP-1-positive mucosal mast cells within the colon and enhanced mMCP-1 levels in the serum suggest a pathophysiological role for mucosal mast cells in this model. In addition, the stool water content of allergic mice was enhanced, reflecting occurrence of diarrhea 24 h after oral allergen challenge. In the casein-sensitized mice, reduced intestinal smooth muscle contraction was observed. This suggests dysmotility of the colon shortly after oral exposure to the allergen. Indeed, constipation and/or diarrhea are among the symptoms in patients affected with CMA. Overall, the tools described in this study might open new avenues to unravel underlying mechanisms of whey and casein allergy in mice.

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