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Antacids and dietary supplements with an influence on the gastric pH increase the risk for food sensitization

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Summary

Background—Elevation of the gastric pH increases the risk for sensitization against food allergens by hindering protein breakdown. This can be caused by acid-suppressing medication like sucralphate, H2-receptor blockers and proton pump inhibitors, as shown in recent murine experimental and human observational studies.

Objective—The aim of the present study was to assess the sensitization capacity of the dietary supplement base powder and of over-the-counter antacids.

Methods—Changes of the pH as well as of protein digestion due to base powder or antacids were measured *in vitro*. To examine the *in vivo* influence, BALB/c mice were fed codfish extract with one of the acid-suppressing substances. Read-out of antibody levels in the sera, of cytokine levels of stimulated splenocytes and of intradermal skin tests was performed.

Results—The pH of hydrochloric acid was substantially increased *in vitro* by base powder as well as antacids in a time- and dose-dependent manner. This elevation hindered the digestion of codfish proteins *in vitro*. A significant increase in codfish-specific IgE antibodies was found in the groups fed codfish combined with Rennie® Antacidum or with base powder; the latter also showed significantly elevated IgG1 and IgG2a levels. The induction of an anaphylactic immune response was proven by positive results in intradermal skin tests.

Conclusions—Antacids and dietary supplements influencing the gastric pH increase the risk for sensitization against allergenic food proteins. As these substances are commonly used in the general population without consulting a physician, our data may have a major practical and clinical impact.

Keywords

antacids; base powder; food allergy; gastric digestion; sensitization against food

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Introduction

The process of sensitization against food proteins and its relevant risk factors are not fully understood yet. Recently, we have shown that the use of anti-acid drugs leads to an enhanced risk for food allergy [1-3]. The underlying reason may be an elevation of the gastric pH. This is caused by different mechanisms depending on the applied drug, like irreversible blocking of the gastric parietal cell function caused by proton pump inhibitors [4], blocking of histamine receptors by H2-receptor blockers/antagonists [5] or by binding of gastric acid (sucralphate, antacids) [6]. As a consequence, the precursor pepsinogen cannot be converted into its active form pepsin, and therefore peptic digestion is inhibited. Additionally, sucralphate has pepsin-binding capacity, which supports the effect of hindering digestion [7, 8]. Furthermore, sucralphate contains aluminium, which independently leads to an increased probability of sensitization when applied parenterally [9] or via the oral route [10].

Apart from these drugs, which are only available on prescription, there are a number of other substances that may increase the gastric pH and therefore promote sensitization. Such substances are for instance over-the-counter selling drugs like the antacids Rennie® Antacidum (Bayer Austria, Vienna, Austria) and Rennie® Digestif (Bayer Austria), which are explicitly made for neutralization of gastric acid. From an oral survey in four different pharmacies in Vienna, we extrapolated the numbers to all pharmacies present in the city and calculated that there were as many as 100 000 packages of the antacid Rennie® Antacidum sold in 2007 in Vienna. In addition to antacids, dietary supplements like base powder can be found in every supermarket. These supplements are taken for supplementation of mineral nutrients and trace elements, and furthermore are broadly consumed as a result of advertisement against the ‘individual’s over-acidification’ due to a disadvantageous daily diet and environmental stress.

In our earlier studies, we have already investigated the impact of an increased pH on the *in vitro* digestion of different food allergens in several simulated gastric fluid (SGF) experiments. For a start, we used 3.2 mg pepsin/mL SGF [2], according to WHO standards and The International Pharmacopeia (http://www.who.int/foodsafety/publications/biotech/en/ec_jan2001.pdf), and later reduced the pepsin concentration to 0.87 mg/mL SGF [3] in order to mimic a more physiological situation. At both concentrations, pepsin was functional at low, but not at elevated pH. Furthermore, we applied either porcine pepsin or pharmaceutical enzyme tablets [11], according to the procedure of Vieths et al. [12], with slight modifications. Taken together, all *in vitro* digestion experiments and *in vivo* animal experiments showed that an appreciable number of typical class I food allergens (i) are not stable to gastric digestion but (ii) are able to induce sensitization [1, 2, 3, 13]. The generated T-helper type 2 (Th2)-dominated milieu could furthermore be transferred to the offspring, when pregnant mice were fed codfish proteins under acid suppression [1]. Accordingly, a human study confirmed that the usage of anti-acids during pregnancy confers an enhanced risk for babies to develop allergies [14].

The aim of the present study was to show whether the above-mentioned over-the-counter substances base powder and Rennie® have an effect on the gastric pH *in vitro*. If so, we aimed to determine, in a BALB/c mouse model, whether this would affect the immune response against a concomitantly applied food allergen, taking codfish as a paradigm.

Materials and methods

Preparation of codfish extract

Commercially available frozen codfish was used to prepare an extract, as described previously [3]. Fresh cod meat was ground in liquid nitrogen and extracted overnight (o.n.) at 4 °C in 10 mM phosphate buffer, 2 mM EDTA, 0.01% pepstatin A and 3 mM NaN_3. After two centrifugation steps (325 **g**, 15 min, 4 °C), the supernatants were dialysed (dialysis membrane cut-off 6000–8000 Da; Spectra/Por, Houston, TX, USA) against distilled water o.n. at 4 °C. The dialysed proteins were frozen at −70 °C and freeze-dried. Extract quality was ascertained by means of SDS-PAGE according to the method of Laemmli [15] by Coomassie brilliant blue staining, and the molecular weight was calculated using pre-stained protein standards (Sigma, Vienna, Austria). The protein concentration of the extract was determined according to the method of Bradford [16] using a Bio-Rad Protein Assay (Bio-Rad, Munich, Germany).

Measurement of pH changes and codfish digestion in vitro

Hydrochloric acid (pH 1.2, 10 mL) was mixed with different amounts (0–110 mg/mL) of either base powder (St. Rodegan®, Pharma Force, Klagenfurt, Austria), Rennie® Antacidum or Rennie® Digestif. Changes in the pH were measured after 90 s at room temperature (RT) (22 °C) and at 37 °C. For investigation of time dependency, 220 mg base powder, Rennie® Antacidum or Rennie® Digestif were added to 10 mL HCl (initial pH 1.2), and pH changes were measured at different time intervals for up to 180 min.

For *in vitro* digestion, 10 mg/mL codfish extract in phosphate-buffered saline (PBS) (50 μL) was added to 200 μL SGF: 0.03 M NaCl, pH 1.2 with 1 M HCl, 0.12 mg/mL pepsin from porcine gastric mucosa (3200–4500 U/mg; Sigma, Steinheim, Germany). This corresponds to a ratio enzyme : substrate of about 1 : 20 (w/w), according to the procedure of Moreno et al. [17], comparable with physiological conditions as assessed by Jones et al. [18]. Codfish in SGF was either mixed with 60 mg/mL of base powder, Rennie® Antacidum or Rennie® Digestif. Mixtures were incubated at 37 °C for different time intervals, separated on 15% SDS-PAGE and stained with Coomassie brilliant blue. Controls (codfish extract in distilled water, codfish in SGF, test substances alone or SGF alone) were incubated at 37 °C for 2 h.

Sensitization protocol of mice

BALB/c mice (female; 6–8 weeks old) were purchased from the Institute of Laboratory Animal Science and Genetics (Medical University of Vienna, Austria) and treated according to European Community rules of animal care with the permission of the Austrian Ministry of Science (BMWF-66.009/0108-C/GT/2007). On days 0/1, 14/15, 21/22, 28/29, 35/36 and 42/43, BALB/c mice ($n = 8$ per group) were immunized intragastrically (for details see Table 1). The amounts of acid-suppressing substances were calculated according to the suggestion for daily intake on labels and adjusted from human to mouse body weight and mouse metabolism (six to eightfold of human). Blood samples were drawn by tail-bleeding on days –1 (pre-immune serum), 6, 13, 20, 27, 34, 41 and 49 and sacrifice was performed on day 50.

Antibody detection in serum by an enzyme-linked immunosorbent assay

Microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with codfish extract (2 μg per well in 100 μL 50 mM $NaHCO_3$, pH 9.6) o.n., 4 °C and blocked with TBST/1% bovine serum albumin (BSA) (RT, 2 h). Serum samples were diluted 1 : 10 for IgE and 1 : 100 for IgG1 and IgG2a detection in TBST/0.1% BSA, and incubated o.n. at 4 °C. Isotype standard antibodies (BD Pharmingen, Schwechat, Austria) were used for dilution series

(starting concentration 500 ng/mL, dilution steps 1 : 2). Isotype-specific rat-anti-mouse antibodies (BD Pharmingen) were used 1 : 700 in TBST/0.1% BSA (RT, 2 h) and peroxidase-labelled anti-rat antibody (GE Healthcare, Buckinghamshire, UK) 1 : 2000 (RT, 2 h). Detection was performed using ABTS (Sigma) and optical density was determined in a microplate reader (Spectra Max Plus 384, Sunnyvale, CA, USA).

Skin tests of mice

On the day of sacrifice, Evans blue (100 μL of 5 mg/mL NaCl 0.9%; Merck, Darmstadt, Germany) was injected into the tail vein of mice. Subsequently, 30 μL of codfish extract (50 μg/mL PBS), 30 μL of hazelnut extract (50 μg/mL PBS) as control allergen, mast cell degranulation compound 48/80 (20 μg/mL PBS; Sigma) as positive control and PBS as negative control were administered intradermally into the shaved abdominal skin. After 20 min, mice were killed and the test area of the skin was prepared. The diameter of the colour reaction was measured on the inside of the abdominal skin. The colour intensity was determined using a hand-held reflection densitometer (Vipdens, Brixen, Italy). Skin reactivity indices were calculated as described previously [19, 20] using the following formula: diameter of skin reactivity×densitometrical signal intensity×number of reactive mice/number of tested mice.

Isolation of splenocytes and detection of cytokines

Mice were killed and the spleens were removed. Spleen cell suspensions of individual spleens were prepared immediately by cutting, mincing and filtering through 70 μm nylon meshes (BD Biosciences, Schwechat, Austria) under sterile conditions. Cells were resuspended in RPMI medium (Gibco®, Invitrogen, Lofer, Austria), supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicilline/streptomycine. Mononuclear cells were isolated by density separation (Lympholyte-M, Cedarlane, Burlington, ON, Canada) according to the manufacturer's instructions. Cells were plated (4×10^5 cells/well) in sterile round-bottom 96-well tissue culture plates (Costar, New York, NY, USA). For stimulation, codfish extract (400 μg/mL), hazelnut extract (400 μg/mL), Con A (5 μg/mL) as positive control or medium were added. Stimulated cells were cultured for 72 h at 37 °C and 5% CO_2. The supernatants were harvested and stored at −20 °C until further use for cytokine evaluation. Measurement of IL-4, IL-5, IL-10, IL-13 and IFN-γ was performed by ELISA with anti-mouse cytokine antibodies and standards (Bender MedSystems, Vienna, Austria) in the supernatants of stimulated splenocytes (diluted 1 : 2), according to the manufacturer's instructions.

Statistics

Statistical comparison of antibody and cytokine levels between groups was performed using Mann–Whitney *U*-test, using the software PASW Statistics (version 18.0 for Windows). Differences were considered statistically significant at *P*-values ≤0.05.

Results

Changes of pH and impact on gastric digestion of base powder and antacids in vitro

The addition of base powder to HCl increased the pH in a dose- (Fig. 1a) and time-dependent (Fig. 1b) manner from pH 1.2 up to 4.0 within 90 s. The pH increase was even more pronounced with the Rennie® substances, as a pH of 5.5 was reached again within 90 s and even increased up to 7.42 within 180 min (Figs 1a and b).

These pH changes also had negative effects on the *in vitro* digestion of codfish extract; whereas codfish was readily digested by SGF after 30 s of incubation, the addition of base powder and Rennie® antacids blocked the digestion of proteins for up to 120 min, which

represents the average gastric transit time (Fig. 2). Especially, the main protein parvalbumin at 12 kDa and the protein around 17 kDa as well as some larger proteins between 35 and 50 kDa were still observed after a 120-min incubation when acid-neutralizing Rennie® substances were added. Parvalbumin digestion was blocked by addition of base powder up to 30 min, and proteins with a molecular weight of around 15 and 40 kDa even remained up to 120 min.

Treatment of mice with codfish in combination with anti-acid substances results in a specific T-helper type 2-associated antibody response

Female BALB/c mice were fed with codfish extract alone, with one of the anti-acid substances alone or with a combination of codfish extract together with one of the anti-acids on two consecutive days per week six times. A significantly higher increase in allergen-specific IgE antibodies was seen in mice treated with codfish/BP (mean±SD: 8.3±5.7 ng/mL) or codfish/RA (mean±SD: 9.4±10.7 ng/mL) compared with the codfish-only group (mean±SD: 3.6±6.6 ng/mL) (Fig. 3). Significantly higher codfish-specific IgG1 antibody levels could be detected in the codfish/BP group (mean±SD: 3.8±2.0 μg/mL) compared with the codfish-only group (mean±SD: 0.9±0.7 μg/mL) (Fig. 3). Further, significantly elevated codfish-specific IgG2a levels were also found after gavages of codfish/BP (mean±SD: 0.7±0.2 μg/mL vs. codfish group: mean±SD: 0.6±0.02 μg/mL) (Fig. 3).

Levels of T-helper type 2 cytokines are elevated in splenocytes of anti-acid-treated mice

Although not statistically significant, a relative elevation of Th2 cytokines was found in the acid-suppressed groups: higher IL-13 levels in the supernatants of splenocytes were observed after stimulation with codfish in codfish/BP, codfish/RA and also in codfish/RD-treated mice, and IL-4 and IL-5 levels were mainly increased after codfish/RA and codfish/RD treatment (Fig. 4). No differences in the mean values were found for IL-10 or IFN-γ levels between groups fed codfish with or without acid-suppressing substances (data not shown). Cytokine levels remained at the base level after stimulation with medium or hazelnut extract, which was used as a control allergen (data not shown).

Skin tests showed a positive reaction after codfish stimulation in anti-acid-treated mice

The *in vivo* effect of the Th2 response was investigated by intradermal skin tests. Increased skin reactivity indices to codfish were observed in mice treated with codfish/BP, codfish/RA and codfish/RD (Fig. 5). No reactions to control substances PBS or hazelnut extract were observed (data not shown).

Discussion

In previous studies, we have shown that anti-acid drugs, only available on prescription by a physician, possess the potential to block gastric digestion of food allergens and consequently elevate the risk for food sensitization [1-3, 21]. The present study aimed to investigate whether the same mechanism also holds true for over-the-counter sub-stances, like the antacids Rennie® Antacidum and Rennie® Digestif, or dietary supplements, like base powder.

Fish is among the most common food allergens, and the prevalence of codfish-specific IgE among 284 children with food hypersensitivity in Sweden was as high as 4% [22]. Therefore, we used codfish as a model protein extract in our studies. Another important aspect is that codfish proteins are usually relatively susceptible to gastric digestion. Therefore, these digestion-labile proteins should less frequently lead to sensitization, but are still among the most potent elicitors of food allergies to be labelled within the European community (http://ec.europa.eu/food/food/labellingnutrition/foodlabelling/

proposed_legislation_en.htm). Our hypothesis is that they may gain sensitization capacity in settings when digestion is impaired. We demonstrated here that indeed inhibition of the gastric digestion and sensitization can be caused by dietary supplements like base powder or by over-the-counter antacids. A study in humans by Atanassoff et al. [23] indeed showed that the gavages of sodium citrate (which is also contained within base powder) to human patients increased the gastric pH up to 6.8 within 2 min, and maintained it above pH 2.5 for up to 6 h. This study implies that our *in vitro* observations could also be true in the human situation. Even more pronounced in our hands was the pH increase up to pH 7.4 caused by the antacids Rennie® Antacidum and Rennie® Digestif. The significant increase of codfish-specific IgE antibodies in the group fed fish protein combined with either base powder or Rennie® Antacidum indicated a shift towards a Th2-type humoral response, which was further supported by the Th2 cytokines IL-4 and IL-5 in the Rennie® Antacidum group, and also by mainly positive reactions in *in vivo* skin tests. These reactions point towards allergic immune responses with biological relevance. In the mouse model, IgE as well as IgG1 may be responsible for such a positive skin reaction [2], as it is already known that specific IgG in complex with its antigen can bind directly to FcγRIII on mast cells and thereby achieve triggering capacity [24, 25]. The serological analyses indicated that Rennie® Digestif induced lower levels of Th2 antibodies IgE and IgG1. This result would fit with the fact that digestive enzymes papain and pancreatin are contained in Rennie® Digestif, which could show some digestive capacity *in vivo* at the elevated pH. The observed hypersensitivity in skin tests might be due to the fact that low levels of IgE can also accumulate in the skin through high-affinity binding to FcεRI-positive cells and elicit hypersensitivity. In any case, Rennie® Digestif was taken off the market in 2007 due to low sale numbers.

In mice fed with codfish concomitant with base powder in addition to IgE and IgG1, IgG2a antibodies also increased significantly. Such mixed Th1/Th2 immune responses were previously observed in BALB/c mice not only when strong adjuvants, e.g. aluminium hydroxide, were given subcutaneously [26], but also under acid-suppressing feeding conditions, when antigen was applied together with the alum-compound sucralphate [9].

The pH elevation by the tested antacids was causative for a blockage of codfish protein digestion, which otherwise would be rapidly degraded under physiological, acidic pH [27]. From our dose-finding experiments, we calculated that the recommended dose per intake during acute symptoms of two tablets of Rennie® Antacidum or Rennie® Digestif or one teaspoon of base powder could considerably increase the pH of gastric juice in humans. These doses correspond to roughly 30 mg/mL, and as Fig. 1 shows, this is a concentration sufficient to increase the pH above the optimum for pepsin [28]. Therefore, the *in vitro* digestion was performed under reasonable conditions perfectly comparable with the human situation. The resulting blockage of digestion was most pronounced under addition of Rennie® Antacidum, in agreement with the observation that this substance also led to the highest increase of the pH. Importantly, also the dietary supplement base powder, which increased the pH up to 4.52 at 37 °C, could inhibit protein breakdown of several codfish proteins around 15 and 40 kDa for up to 2 h.

There are only sporadic reports of impaired digestion and sensitization in the literature; however, Michael and Jain [29, 30] showed already in the 1990s that sensitization to ovalbumin (OVA) or BSA only occurs when gastric digestion is circumvented by application of the antigens directly into the ileum. Furthermore, it is long known among immunologists that oral antigen feedings lead to antibody responses when acid is suppressed concomitantly (e.g. by sodium bicarbonate) [31]. In addition, in the studies of Michael and colleagues mentioned above, OVA was not able to induce an immune response when digested before oral application, even when the remaining fragments were applied concomitant with the anti-acid drug cimetidine. These studies perfectly match our

observations and further strengthen our hypothesis that gastric digestion has a gate-keeping function for allergy prevention by starting the breakdown of ingested food proteins. With the acidic pH of the chime (pH<4.5), the stomach also provides the necessary signal for the consecutive stimulation of the pancreas for the release of its enzymes, especially via secretin in the proximal duodenum. This implies that an increased pH of the stomach or – in other words – the lack of normal, physiologically low pH of the stomach, as is the case, e.g. after Billroth I/II surgery, may subsequently also result in impaired pancreatic digestion, i.e. maldigestion [32].

In contrast to codfish, many other 'true' food allergens are actually stable to gastric digestion and/or food processing like heat treatment [33]. Several additional proteins may cause symptoms due to cross-reactivity only, with the primary sensitizer being inhaled, because they are quickly degraded by pepsin at a low pH [20]. In addition to the stability of a protein, other factors may determine its sensitizing and allergen-triggering capacity, for instance, the likelihood of the formation of dimers or multimers [34, 35]. Additionally, the genetic predisposition [36], the environmental influence like diesel-exhaust particles (reviewed in Bartra et al. [36]), the hygienic status and the route of contact with the food – either via skin or via ingestion [37], the food matrix (reviewed in Nowak-Wegrzyn and Fiocchi [38]), the method of food processing (e.g. pasteurization of milk [39]) and the dosage of a food protein taken in during conditions making individuals susceptible to sensitization [13] may – alone or in concert – influence the allergenic potential of a protein. Consecutively, immune cells in the gut are confronted with immunogenic particles, which present a 'danger signal'. Therefore, these cells induce an immune response rather than tolerance or ignorance.

In summary, dietary supplements like base powder and antacids increase the gastric pH and, therefore, increase the risk for sensitization against food allergens, even to those that are usually digestion labile. The underlying mechanism may be the hindrance of protein digestion, resulting in larger protein leftovers, which are able to induce an immune response rather than tolerance. Keeping in mind the widespread usage of dietary supplements (like base powder) and over-the-counter antacids world-wide, the present study may offer an additional explanation for the increasing/increased numbers of food allergies in the last decades.

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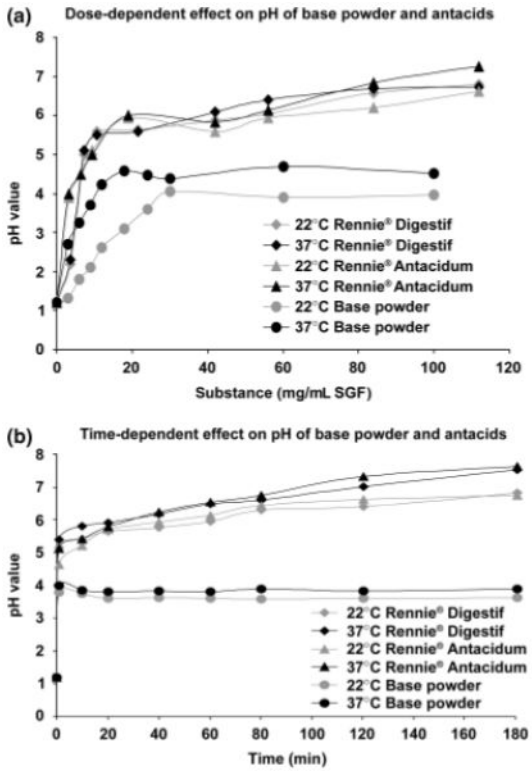

Fig. 1.

(a) Dose-dependent and (b) time-dependent change of the pH after addition of base powder and antacids. Hydrochloric acid (pH 1.2, 10 mL) was mixed with (a) different amounts of base powder, Rennie® Antacidum or Rennie® Digestif or (b) an amount of 22 mg/mL of each substance and the pH measured at different time intervals.

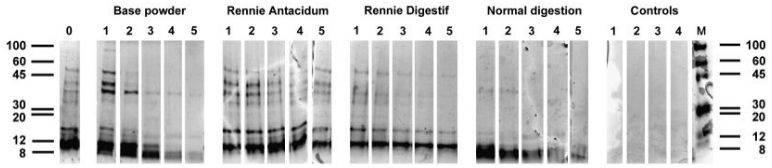

Fig. 2.

Digestion of codfish proteins is hindered under elevated pH. The incubation of codfish extract in simulated gastric fluid (SGF) with base powder, Rennie® Antacidum or Rennie® Digestif for (1) 30 s, (2) 10 min, (3) 30 min, (4) 60 min or (5) 120 min reveals protein preservation for up to 2 h, in contrast to rapid digestion under normal acidic conditions. Codfish was not digested when incubated with distilled water (0) for 120 min. (M) standard protein marker. Controls: (1) base powder in SGF; (2) Rennie® Antacidum in SGF; (3) Rennie® Digestif in SGF; and (4) SGF.

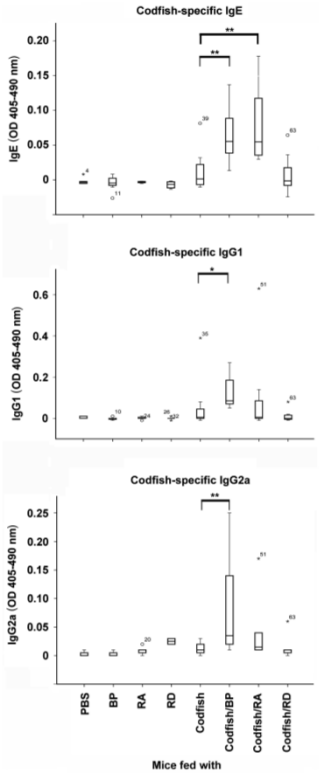

Fig. 3.

Codfish-specific antibodies increase during feedings under concomitant acid suppression. Significantly elevated IgE, IgG1 and IgG2a levels in the last immune serum (after 6×2 feedings) reveal an enhanced immune response in groups fed codfish together with either base powder (codfish/BP) or with Rennie® Antacidum (codfish/RA). The OD values of pre-immune sera were subtracted from the values of immune sera. The boxes represent the range of the inner quartiles of the samples divided by the median. Sera with signals showing more than a 1.5-fold deviation from the end of the box were defined as outlines and marked as circles. Sera with titres lying more than threefold away were defined as extremes and marked with asterisks. Brackets indicate the groups that are statistically significantly different compared with the codfish-only group (*$P \leq 0.05$, **$P \leq 0.01$).

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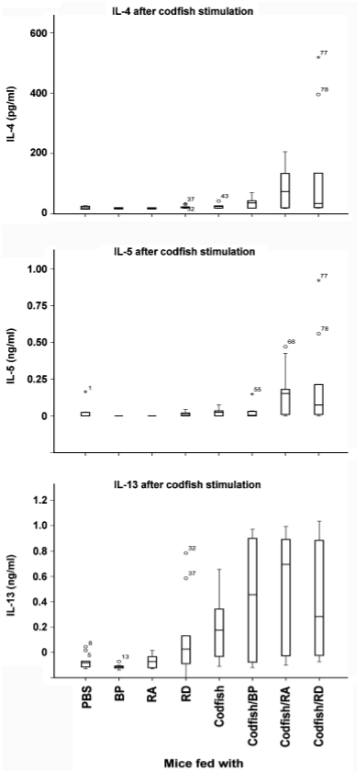

Fig. 4.

Cytokine levels confirm the bias towards a T-helper type 2 reponse. Th2 cytokine levels of IL-4, IL-5 and IL-13 are elevated (although not significantly) in splenocyte supernatants after stimulation with codfish extract in mouse groups subjected to acid suppression with Rennie® substances during codfish feedings. The boxes represent the range of the inner quartiles of the samples divided by the median. Sera with signals showing more than a 1.5-fold deviation from the end of the box were defined as outlines and marked as circles. Sera with titres lying more than threefold away were defined as extremes and marked with asterisks.

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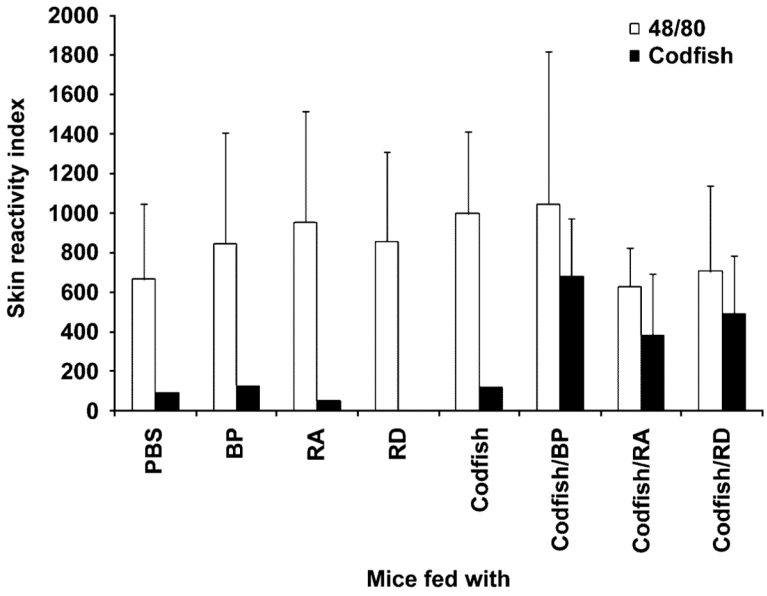

Fig. 5.

Positive reactions in intradermal skin tests underline the biological relevance of the humoral and cellular allergic response towards a food allergen when combined with anti-acid substances. The most intensive reactions to codfish were found in the groups treated with codfish together with either base powder, Rennie® Antacidum or Rennie® Digestif. Positive controls were performed with compound 48/80, and negative controls (data not shown) with phosphate-buffered saline and the control allergen hazelnut extract. Skin reactivity indexes were calculated as the diameter of skin reactivity×densitometrical signal intensity×number of reactive mice/number of tested mice.

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Table 1

Intragastric immunization of mice

Group name	Substances applied in 200 mL/mouse/ gavage
Phosphate-buffered saline (PBS)	PBS
BP	Base powder (70 mg) in PBS
RA	Rennie® Antacidum (25 mg) in PBS
RD	Rennie® Digestif (25 mg) in PBS
Codfish	Codfish extract (2 mg) in PBS
Codfish/BP	Codfish extract (2 mg) and BP (70 mg) in PBS
Codfish/RA	Codfish extract (2 mg) and RA (25 mg) in PBS
Codfish/RD	Codfish extract (2 mg) and RD (25 mg) in PBS