

ORIGINAL PAPER

The impact of aluminium in acid-suppressing drugs on the immune response of BALB/c mice

R. Brunner*, J. Wallmann*, K. Szalai*, P. Karagiannis*, T. Kopp†, O. Scheiner*, E. Jensen-Jarolim* and I. Pali-Schöll*

*Department of Pathophysiology, Medical University of Vienna, Vienna, Austria and †Department of Dermatology, Medical University of Vienna, Vienna, Austria

Clinical and Experimental Allergy

Summary

Background Recently we have shown that anti-acid drugs lead to an enhanced risk of food allergy. This may be due to hindered peptic digestion, caused by an elevation of the gastric pH. Additionally, it is known that aluminium-linked antigens lead to an increased probability of sensitization.

Objective Our aim in this study was to show whether sucralfate promotes sensitization not only by preventing peptic digestion but also by acting as a T-helper type 2 (Th2) adjuvant.

Methods To avoid the effect of sucralfate on the gastric pH and to show only the adjuvant effect, BALB/c mice were immunized on the parenteral route with codfish extract plus sucralfate, and control groups with aluminium hydroxide (alum) (Th2 adjuvant) or monophosphoryl lipid A (MPL) (Th1 adjuvant). Antigen-specific antibodies and cytokine levels were determined. The *in vivo* effect was investigated by intradermal skin tests.

Results Codfish-specific high IgG1 and IgE antibody levels as well as elevated IL-4 and IL-5 levels in alum- and MPL-treated mice, but more importantly also in sucralfate-treated mice, indicated a Th2 shift. Positive skin tests confirmed this Th2 response.

Conclusions Our data show that parenterally applied sucralfate is able to induce a Th2 response probably due to the aluminium content. This indicates that orally applied sucralfate may lead to an enhanced risk of food allergy not only by inhibiting peptic digestion but also by acting as a Th2 adjuvant.

Keywords adjuvant, aluminium, food allergy, sensitization against food, sucralfate

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Correspondence:

I. Pali-Schöll, Department of Pathophysiology, Medical University of Vienna, Vienna, Austria.
E-mail: isabella.pali@meduniwien.ac.at

Introduction

The prevalence of food allergy has been steadily increasing during the last decades, especially in western countries. Currently about 6% of children and about 3.5–4% of adults in the United States are affected [1, 2]. No conclusive explanation of this development has been brought, although there is great consensus that the risk of acquiring food allergies depends on genetic predisposition, increased exposure to allergens [3, 4] and several environmental factors [5]. Basically there are three mechanisms for sensitization to food allergens. First, adverse reactions to food can be a consequence of cross reactivity to airborne allergens. Second, a direct sensitization can occur in the gastrointestinal tract via the oral route. The latter is believed to require an intact form of food antigens and resistance to gastric digestion [6, 7]. Last, there is increasing evidence that exposure through the skin may

be a route of sensitization to food allergen as well [8–10]. The process of sensitization and its relevant factors are not fully understood yet, but recently we have shown that the use of anti-acid drugs leads to an enhanced risk of food allergy [11–13]. This may be due to an elevation of the gastric pH, which is caused either by irreversible blocking of the gastric parietal cell function (proton pump inhibitors), blocking of histamine receptors (H2-receptor blockers) or by binding of gastric acid (sucralfate, antacids) [14]. As a consequence, pepsinogen cannot be converted into its active form pepsin and peptic digestion is inhibited. Additionally, sucralfate has pepsin-binding capacity, which supports the effect of hindered digestion [15, 16].

Apart from that, it is known that aluminium-linked antigens lead to an increased probability of sensitization [17]. Sucralfate is an aluminium-containing drug for reflux treatment [18, 19]. Our aim in this study was to show whether sucralfate promotes sensitization not only

by preventing peptic digestion but also by acting as a T-helper type 2 (Th2) adjuvant. To avoid the effect of blocked gastric digestion on the process of sensitization and to show only the adjuvant effect, we applied sucralfate on the parenteral route, in combination with codfish extract as model allergen in BALB/c mice. Subsequently, immune responses were monitored on the humoral as well as on the cellular level.

Materials and methods

Preparation of codfish extract

Commercially available frozen codfish was used to prepare an extract, as described previously [12]. Fresh cod meat was grounded in liquid nitrogen and extracted overnight at 4 °C in 10 mM phosphate buffer, 2 mM EDTA, 0.01% pepstatin A and 3 mM Na₂S₂O₃. After two centrifugation steps (325 g, 15 min, 4 °C), supernatants were dialysed (dialysis membranes cut-off 6000–8000; Spectra/Por, Houston, TX, USA) in distilled water overnight at 4 °C. The dialysed proteins were frozen at –70 °C and freeze-dried. Extract quality was confirmed by means of SDS-PAGE according to the methods of Laemmli [20] by Coomassie brilliant blue staining, and the molecular weight was calculated using pre-stained protein standards. The protein concentration of the extract was determined according to the method of Bradford [21] using a Bio-Rad Protein Assay (Bio-Rad, Munich, Germany).

Protocol of sensitization of mice

BALB/c mice (female: 6–8 weeks) were purchased from Charles River GmbH (Sulzfeld, Germany) and treated according to European Community rules of animal care with the permission of the Austrian Ministry of Science (GZ 66.009/0033-BrGT/2005). On days 0, 7 and 21, BALB/c mice ($n=5$ per group) were immunized subcutaneously with either codfish extract [20 µg codfish/50 µL phosphate-buffered saline (PBS)] plus 100 µL sucralfate (1 g sucralfate/5 mL suspension; Ulcogant, Merck Vienna, Austria); codfish extract (20 µg codfish/50 µL PBS) plus 100 µL 2% aluminium hydroxide (alum) (Sigma, Steinheim, Germany); or codfish extract (20 µg codfish/50 µL PBS) plus 100 µL monophosphoryl lipid A (MPL) (Sigma). Control groups were immunized either with these adjuvants with PBS instead of codfish or with codfish extract (20 µg codfish/50 µL PBS) plus 100 µL PBS. Blood samples were drawn by tail-bleeding on days 0 (pre-immune serum), 7, 21, 28, 36, 56 and 70.

Antibody detection in serum by enzyme-linked immunosorbent assay

Microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with codfish extract or hazelnut extract

(20 µg per well in 100 µL 50 mM NaHCO₃, pH 9.6). Serum samples were diluted 1 : 100 for IgG1 and IgG2a, or 1 : 10 for IgE. Further ELISA procedure was performed as described previously [22].

Skin tests of mice

On the day of killing, 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 irrelevant control allergen, mast cell degranulation compound 48/80 (20 µg/mL PBS; Sigma) as positive control or PBS as negative control were administered intradermally into shaved abdominal skin. After 20 min mice were killed and skinned. A positive response can be seen as a blue colour reaction on the inside of the abdominal skin due to vascular leakage.

Isolation of splenocytes and detection of cytokines

Mice were killed and spleens were removed. Spleen cell suspensions of individual spleens were prepared immediately by cutting, mincing and filtering through 70 µm nylon meshes. Cells were resuspended in RPMI medium (Gibco[®], Invitrogen, Lofer, Austria), supplemented with 10% foetal calf serum (FCS), 1% L-glutamine and 1% penicillin/streptomycin. Mononuclear cells were isolated by density separation (Lympholyte-M; Cedarlane, Ontario, Canada) according to manufacturer's instructions. Cells were plated (4×10^6 cells/mL) in sterile round-bottom 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. The cells were cultured for 72 h at 37 °C and 5% CO₂. Supernatants were harvested and stored at –20 °C, until further use for cytokine measurements. Measurement of IL-4, IL-5 and IFN- γ was performed by ELISA with anti-mouse cytokine antibodies and standards (Bender MedSystems, Vienna, Austria) in supernatants of stimulated splenocytes (diluted 1 : 2), according to manufacturer's instructions.

β -hexosaminidase secretion assay from rat-basophile leukaemia cells

Rat-basophile leukaemia (RBL-2H3) cells (generously provided by Dr Arnulf Hartl, Institute of Biochemistry, University of Salzburg, Salzburg, Austria) were cultured in RPMI medium supplemented with 10% FCS, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES, 1% penicillin/streptomycin and 100 µM 2-mercaptoethanol at 37 °C and 5% CO₂. RBL cells (4×10^4 cells/well in 100 µL medium) were seeded in a 96-well culture plate (96 MicroWell, Nunclon) and incubated overnight at

37 °C and 5% CO₂. Cells were passively sensitized with pooled sera from mice (diluted 1:25 or 1:50 in 50 µL Tyrode's buffer/well) for 2 h at 37 °C and 5% CO₂. Afterwards, cells were washed twice with Tyrode's buffer (137 mM NaCl, 2.68 mM KCl, 1.4 mM CaCl₂, 1.7 mM MgCl₂, 5 mM D-glucose, 10 mM HEPES, 0.42 mM NaH₂PO₄ × H₂O, 12 mM NaHCO₃) at pH 7.2, supplemented with 0.1% bovine serum albumin (Sigma). Codfish extract, hazelnut extract as irrelevant control allergen or ionomycin as positive control were diluted in Tyrode's buffer and added to the wells (0.1 µg in 100 µL/well) for 30 min at 37 °C and 5% CO₂. As a negative control RBL-cells were incubated with Tyrode's buffer without allergens to calculate spontaneous release. Cell lysis was performed by addition of 10 µL of 10% Triton-X (Sigma) in Tyrode's buffer at 37 °C for 5 min for the total release of β-hexosaminidase (100% release). Of the supernatants, 50 µL were discarded and instead 50 µL of assay solution (5 mL 0.1 M citric acid, pH 4.5 with NaOH, mixed with 80 µL 4-methyl-umbelliferyl-N-acetyl-β-D-glucosaminide; Sigma) at pH 4.5 were added. Cells were incubated for 1 h at 37 °C and 5% CO₂, and the reaction was terminated by addition of 100 µL glycine buffer (200 mM glycine, 200 mM NaCl, pH 10.7). Finally, the fluorescence was read at 360/465 nm and the results were calculated as percentage of total release.

Histological preparation of injection sites

Sites of the last subcutaneous injection (on day 21) were marked and punch biopsies of the skin were taken on day 70 (killed). Biopsies were fixated in 7.5% formalin (Brenntag, Vienna, Austria) and embedded in paraffin (UltraPar, J. T. Baker, Deventer, the Netherlands; Sakura Tissue Tek, Sanova, Vienna, Austria). Paraffin sections of 4 µm slices were prepared and stained with haematoxylin (Papanicolaous, Merck) and eosin (1% phloxin B, 1% fuchsintrisol-fonate, 2% eosin B; Merck). Evaluation was performed under a light optical microscope (Axiophot, Zeiss, Germany). Pictures were taken at a magnification of ×4.

Statistical analysis

Statistical comparison of antibody and cytokine levels between groups was performed by the Mann-Whitney *U* test, using the software SPSS (version 14.0 for Windows). Differences were considered statistically significant at *P*-values <0.05.

Results

Treatment of mice with sucralfate as adjuvant results in a specific T-helper type 2 response

Female BALB/c mice were immunized subcutaneously on days 0, 7 and 21 with either codfish/sucralfate, codfish/

alum or codfish/MPL; control groups with adjuvant or codfish only. Mice treated with codfish/sucralfate or codfish/alum showed a significant increase of codfish-specific IgE antibodies after the second and third immunization, indicating that these adjuvants induce a Th2 response. The codfish/MPL-treated group showed a moderate increase of codfish-specific IgE antibodies. The codfish/PBS-treated group and control groups did not show an increase of codfish-specific IgE (Fig. 1a). Mice treated with codfish/sucralfate, codfish/alum or codfish/MPL showed a significant increase of codfish-specific IgG1 antibodies after the second and third immunization (Fig. 1b). All adjuvant-treated groups showed a significant increase of codfish-specific IgG2a antibodies after the third immunization (Fig. 1c). The presence of biologically relevant codfish-specific IgE in codfish/sucralfate (Fig. 2a) and codfish/alum (Fig. 2c)-treated groups at day 70 was further shown in an RBL assay, using pooled mice sera. Control groups treated with PBS/sucralfate (Fig. 2b), PBS/alum (Fig. 2d), codfish/PBS (Fig. 2g), PBS/MPL (Fig. 2f) as well as codfish/MPL (Fig. 2e) did not show codfish-specific IgE. No immune reactions to the control allergen hazelnut extract were seen, either on the antibody-level in ELISA or in RBL assay (data not shown).

Generation of interleukin-4 and interleukin-5 is increased in splenocytes from sucralfate-treated mice

A significant elevation of IL-4 and IL-5 in supernatants of splenocytes was observed not only after stimulation with codfish in codfish/sucralfate and codfish/alum, but also in codfish/MPL-treated mice. This suggests a clear Th2 shift in all adjuvant-treated groups regarding the T-cell activation state (Fig. 3a and b). Results of IFN-γ analysis revealed a moderate, though not significant, elevation in codfish/MPL-treated mice (Fig. 3c). Cytokine levels remained on base level after stimulation with hazelnut extract, which was used as control allergen (data not shown).

Intradermal skin tests showed a positive reaction after codfish stimulation in sucralfate-treated mice

The *in vivo* effect of the Th2 response was investigated by intradermal skin tests. Positive skin test reactions to codfish were seen in mice treated with codfish in combination with sucralfate (Fig. 4a), alum (Fig. 4d) or MPL (Fig. 4f). In contrast, treatment with codfish/PBS (Fig. 4b), PBS/sucralfate (Fig. 4c) and PBS/alum (Fig. 4e), as well as PBS/MPL (Fig. 4g), did not result in a positive skin reaction after codfish stimulation.

Granuloma formation in sucralfate-treated mice

Huge granulomas were already seen macroscopically in groups treated with either sucralfate or alum, in

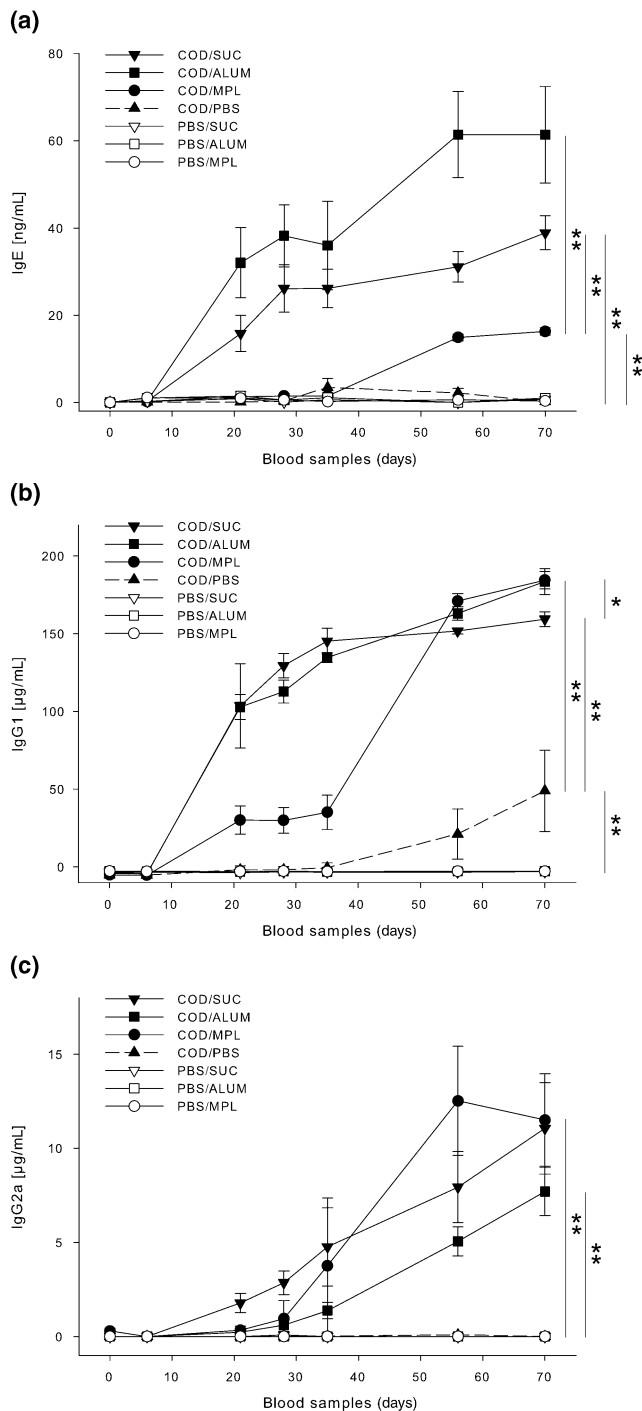


Fig. 1. Codfish/sucralfate treatment causes a Th2 antibody response. (a) Mice ($n = 5$ per group) treated with codfish/sucralfate (COD/SUC) or codfish/alum (COD/ALUM) show a strong increase of codfish-specific IgE antibodies after the second (day 7) and third (day 21) immunization, indicating that these adjuvants induce a Th2 response. In the codfish/PBS (COD/PBS)-treated group and in control groups no increase of codfish-specific IgE is seen. (b) Mice treated with COD/SUC, COD/ALUM or codfish/MPL (COD/MPL) present a strong increase of codfish-specific IgG1 after the second and third immunization. (c) Mice treated with COD/SUC, COD/ALUM or COD/MPL show a strong increase of codfish-specific IgG2a after the third immunization, in contrast to COD/PBS-treated mice and further control groups. * $P < 0.05$, ** $P < 0.01$.

combination with codfish or PBS. For microscopic investigation of injection sites, punch biopsies of cutaneous and subcutaneous tissues were taken, haematoxylin/eosin (H&E) stained and examined histologically. Biopsies of codfish/sucralfate-injected mice showed a typical granuloma with surrounding macrophages (Fig. 5a). The granulomatous reaction was comparable to the results obtained in groups treated with PBS/sucralfate (Fig. 5b), codfish/alum (Fig. 5d) and PBS/alum (Fig. 5e). In contrast, biopsies of codfish/PBS-treated mice showed normal skin with regular epidermis and dermis (Fig. 5c).

Discussion

We have shown previously that anti-acid treatment is an important factor for the induction of food-specific IgE in adult humans, and confirmed this observation in mouse models [11–13, 23]. This effect is most probably caused by an increased gastric pH. As pepsinogen cannot be converted into its active form pepsin, the protein digestion is reduced or even blocked. These studies, including human data, therefore suggest that acid-suppressing drugs are responsible for an enhanced risk of food allergy induction.

Among anti-acid drugs, sucralfate is a common medication for the treatment of gastroesophageal reflux disease in humans. It is a complex of sucrose octasulphate and aluminium hydroxide and provides mucosal protection by creating an adherent complex with the protein exudates of the oesophageal/gastric mucosa. It also has pepsin-, proton- and bile acid-binding capacities that enhance tissue resistance [14, 15, 24]. Therefore, in addition to the elevation of the pH, sucralfate supports the blocking of peptic digestion by binding pepsin directly. Aluminium hydroxide itself is widely used as an experimental and clinical adjuvant in vaccine formulations. It is known that aluminium-linked antigens lead to an increased probability of sensitization and induction of Th2 cell responses [17]. The potentiation of the immune response to antigens is caused by an adsorption to the adjuvant. The major mechanisms responsible for the adsorption of antigens by aluminium-containing adjuvants are electrostatic attraction, hydrophobic attraction and ligand exchange [25]. One of the mechanisms, which have been proposed to explain how aluminium-containing adjuvants potentiate the immune response, is that they convert the soluble antigen into a particulate form and thereby stabilize the conformation [26]. Therefore, we hypothesized in the present work that sucralfate with its alum content enhances the risk of food allergy not only by preventing gastric digestion but also by acting as a Th2 adjuvant.

To avoid the effect of blocked gastric digestion on the process of sensitization and to show only the adjuvant effect, we applied sucralfate on the parenteral route, in combination with codfish extract as model allergen. Our

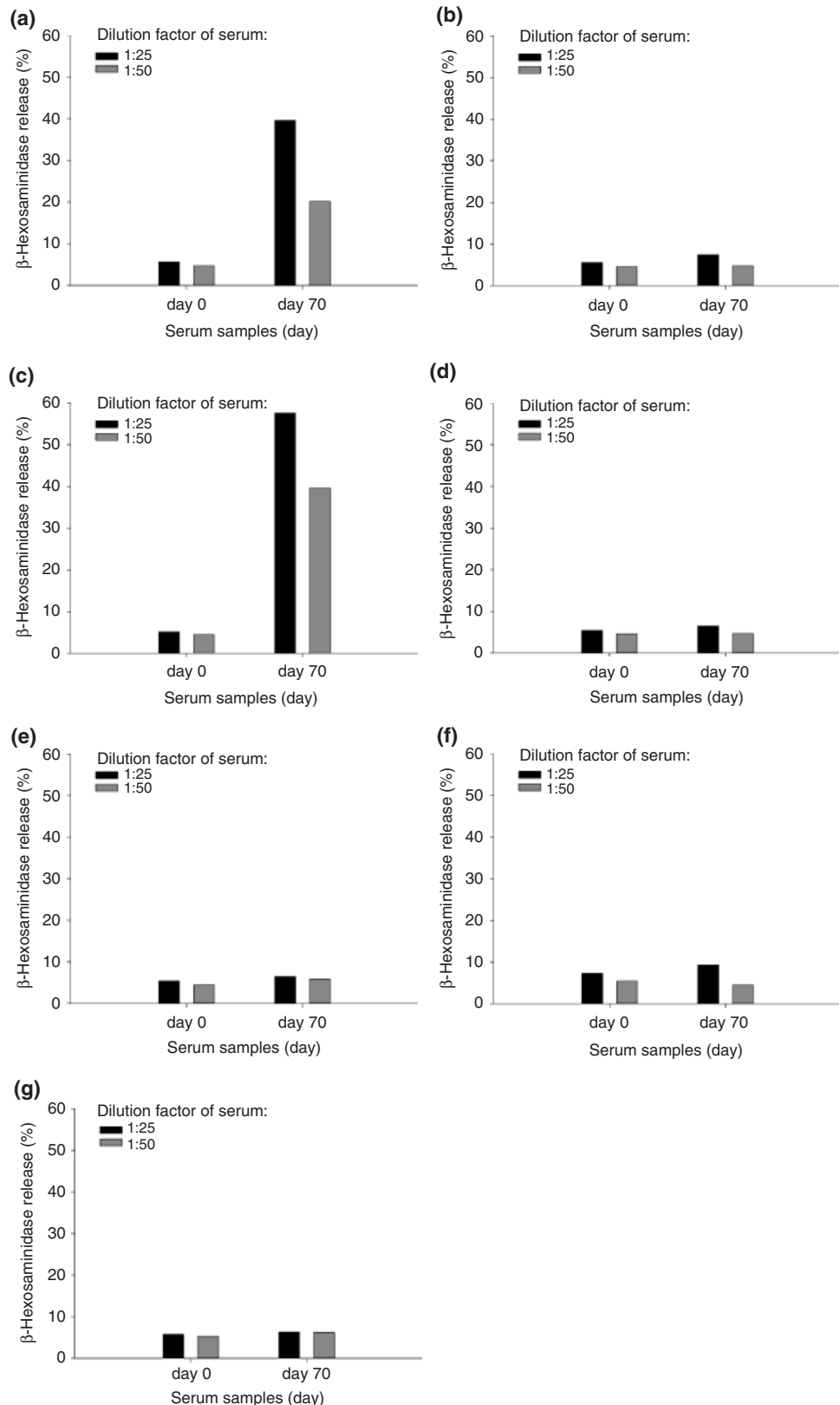


Fig. 2. Codfish-specific IgE with biological relevance in mice is induced after immunization with codfish/sucralfate (a) and codfish/alum (c), but not with PBS/sucralfate (b), PBS/alum (d), codfish/MPL (e), PBS/MPL (f) and codfish/PBS (g). Rat-basophile leukaemia cells were passively sensitized with pooled sera of mice (diluted 1:25 and 1:50) before (day 0) and after the third immunization (day 70). After stimulation with codfish extract, release of β -hexosaminidase (means of triplicates) as percentage of total release with Triton-X (=100%) was determined. Negative controls: Tyrode's buffer or hazelnut extract; positive controls with ionomycin (data not shown). Measurement of fluorometrical intensity (360/465 nm) was performed. No statistical analysis could be performed due to the usage of pooled sera.

BALB/c mouse model showed a significant increase of codfish-specific IgE and IgG1 antibodies, suggesting a clear Th2 shift in codfish/sucralfate as well as in codfish/alum-immunized mice. The specificity of this immune reaction was confirmed in tests of serum on hazelnut

extract, used as control allergen, where no elevation of antibodies was observed. The biological relevance and high specificity of induced codfish-specific IgE in this study was shown in an RBL assay. Cross-linking of antigen-specific IgE antibodies and therefore release of β -hexosaminidase by activated RBL cells was seen after stimulation with codfish extract in codfish/alum and codfish/sucralfate-treated mice at sera dilutions of 1:25 and 1:50. Altogether RBL results confirm our results shown in ELISA. The Th2 bias of the antibody pattern was confirmed by determination of cytokines in spleen cell supernatants after codfish stimulation. Compared to control groups, significantly elevated IL-4 and IL-5 levels were measured in codfish/sucralfate- as well as in codfish/alum-treated mice. IFN- γ remained at low levels in all groups, even in codfish/MPL-treated mice. Although MPL is described as a Th1 inducing adjuvant [27], relatively low levels of IFN- γ and codfish-specific IgG2a antibodies were seen in our model, very similar to the results found in BALB/c mice by other groups [28]. This may be due to the Th2 background of BALB/c mice [29], although a Th1 response in BALB/c mice can be provoked by certain chemicals, like levamisole, under defined conditions [30]. However, such treatment is not completely comparable to our protein immunization experiments.

The *in vivo* relevance of our data was shown by performing intradermal skin test. Positive skin reactions to codfish underlined the results from serological assays. Macroscopical and histological examination of cutaneous and subcutaneous tissue showed granuloma formation at injection sites in sucralfate- and aluminium-treated mice. This was already observed in our earlier studies [13] and is most probably ascribed to the foreign, non-degradable material alum [31].

Conclusion

Our data show that aluminium in parenterally applied sucralfate acts as Th2 adjuvant. This indicates that orally applied sucralfate leads to an enhanced risk of food allergy, not only by inhibiting peptic digestion but also by acting as an adjuvant.

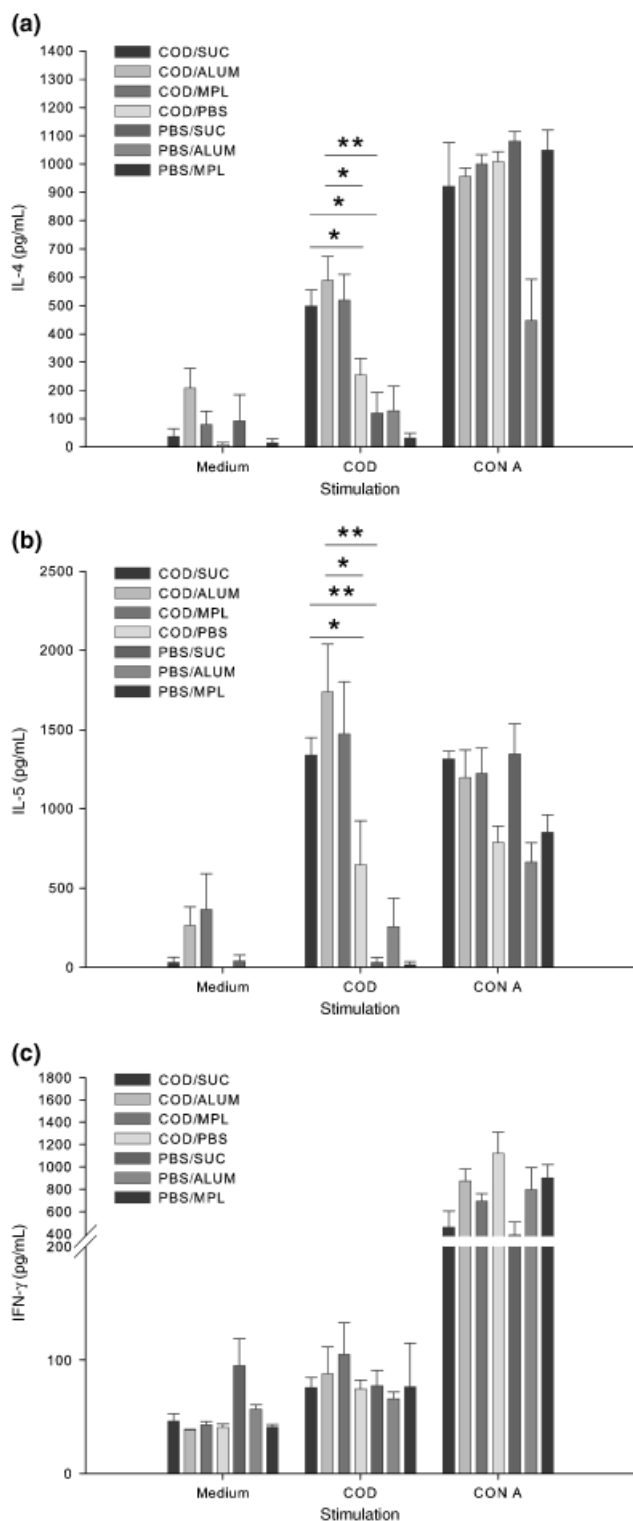


Fig. 3. Codfish/sucralfate treatment causes a Th2 response regarding cytokine levels in spleen cell supernatants. Confirming the Th2 bias of the antibody pattern, codfish/sucralfate (COD/SUC), codfish/alum (COD/ALUM) and codfish/MPL (COD/MPL)-treated mice show high IL-4 and IL-5 levels (a and b) after the stimulation of spleen cells with codfish extract. The codfish/PBS (COD/PBS) and further control groups present significantly lower levels of IL-4 and IL-5. (c) A moderate, though not significant, elevation of IFN- γ is seen in COD/MPL-treated mice. Negative controls: medium; positive control: concanavalin A (CON A). * $P < 0.05$, ** $P < 0.01$.

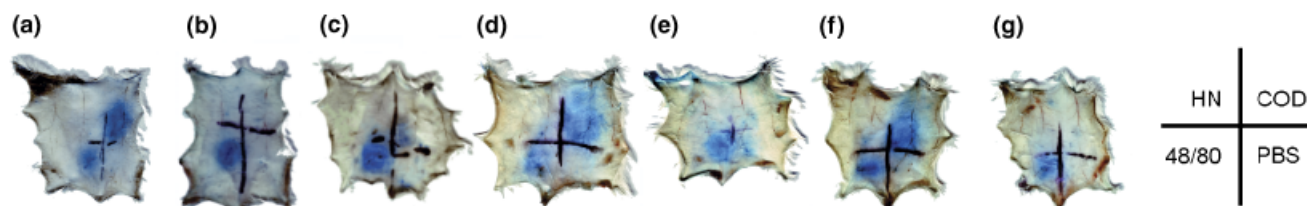


Fig. 4. Codfish/sucralfate-treated (a) as well as codfish/alum-treated (d) and codfish/monophosphoryl lipid A-treated (f) mice ($n = 5$ per group) show a positive reaction to codfish (COD) in type I skin tests. No reaction to codfish is seen in mice fed with either PBS/sucralfate (b), PBS/alum (e), PBS/MPL (g) or codfish extract alone (c), respectively, to the control allergen hazelnut (HN) or PBS. Compound 48/80 was used as positive control. Representative skin tests of each group are shown.

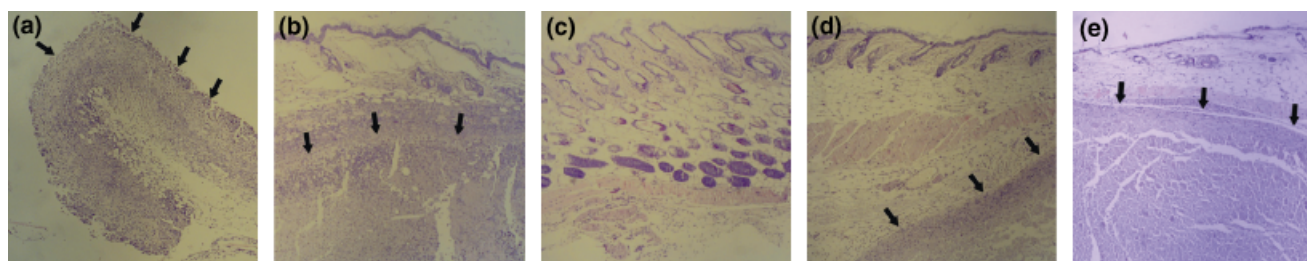


Fig. 5. Codfish/sucralfate- (a) treated mice show granuloma formation, epithelioid macrophages and giant cells, as do mice treated with PBS/sucralfate (b), codfish/alum (d) and PBS/alum (e). Mice treated with codfish extract without adjuvant (c) have regular skin without granuloma.

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