

ORIGINAL ARTICLE

Diclofenac enhances allergic responses in a mouse peanut allergy model

M. Bol-Schoenmakers¹, R. Bleumink¹, M. Marcondes Rezende^{1,2}, E. Mouser¹, I. Hassing¹, I. Ludwig^{1,3}, J. J. Smit^{1,2} and R. H. H. Pieters¹¹Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands, ²Utrecht Center for Food Allergy, Utrecht, The Netherlands and ³TiPharma, Leiden, The NetherlandsClinical &
Experimental
Allergy

Summary

Background Diclofenac and other non-steroidal anti-inflammatory drugs (NSAIDs) interfere with cyclo-oxygenase-mediated synthesis of prostaglandins, resulting in the inhibition of inflammatory immune responses. In contrast, it is known that NSAIDs are able to induce gastrointestinal damage.

Objective Our aim was to investigate whether NSAIDs are able to enhance sensitization or abrogate tolerance to food antigens.

Methods Mice were exposed to diclofenac and sensitized to peanut using cholera toxin as a mucosal adjuvant. In a tolerance model, oral tolerance was induced via feeding of peanut 3 weeks before sensitization with peanut. Diclofenac was administered before peanut feeding. After 4 weeks, peanut-specific antibodies in the serum and cytokine production in the spleen were measured. Induction of intestinal damage after oral exposure with diclofenac and peanut + cholera toxin was examined microscopically.

Results Diclofenac-exposed animals showed increased levels of peanut-specific IgG1, IgG2a and IgE in the serum compared with vehicle-treated animals. Furthermore, peanut-induced cytokine production in the spleen was elevated upon diclofenac treatment. Importantly, diclofenac did not induce peanut-allergic responses in the absence of the cholera toxin, although exposure to diclofenac and peanut + cholera toxin resulted in intestinal epithelial damage. Reduced peanut-specific antibody production in the case of oral tolerance was not reversed after diclofenac exposure. However, oral tolerance, as measured by inhibition of peanut-specific cytokine responses, was reverted by diclofenac.

Conclusions These data point towards an increased risk for induction of food-allergic responses by diclofenac, when other circumstances are also in favour of induction of allergy.

Keywords cyclo-oxygenase, diclofenac, mouse allergy model, non-steroidal anti-inflammatory drugs, oral tolerance, peanut allergy

Submitted 22 October 2009; revised 11 June 2010; accepted 6 July 2010

Correspondence:

M. Bol-Schoenmakers, Utrecht University, IRAS, ITX, P.O. box 80177, NL 3508 TD Utrecht, The Netherlands.
E-mail: m.bol-schoenmakers@uu.nl

Introduction

Food allergy is reported in up to 8% of children below 3 years in the United States, and approximately 2% of the adults experience food-allergic reactions. However, the risk factors that are involved in the induction of food-allergic responses remain obscure [1]. The usual response to orally ingested antigens in the intestine is the induction of tolerance, but the presence of an adjuvant or disturbance of the epithelial barrier function can result in adverse reactions [2, 3]. For instance, the use of non-steroidal anti-inflammatory drugs (NSAIDs) occasionally enhances wheat- or shrimp-induced anaphylaxis [4], and

impairs oral tolerance induction to β -lactoglobulin [5] and ovalbumin [6]. A recent study shows that diclofenac is able to induce sensitization to a co-injected antigen (TNP-Ovalbumin) accompanied by antigen-specific antibody responses [7]. It is therefore of interest to investigate whether the administration of diclofenac is able to elicit or enhance the response towards food proteins.

NSAIDs, like diclofenac, are originally designed to inhibit inflammation by inhibition of cyclo-oxygenase (COX) enzymes. COX enzymes (constitutive COX-1 and inducible COX-2) catalyse the conversion of membrane-derived arachidonic acid into biologically active molecules (prostanoids) like prostaglandins (e.g. PGE₂, PGD₂)

thromboxanes [8] and resolvins [9]. COX-1 is responsible for basal prostanoid synthesis, whereas COX-2 appears to be especially important in inflammatory processes in peripheral tissues. In general, reduction in prostaglandin production results in control of inflammation, pain and fever [10]. However, prostaglandins also have important regulatory effects on the maturation and migration of antigen-presenting cells (APC) and on the secretion of cytokines [8]. For instance, PGE₂ inhibits the production of IL-12 by dendritic cells (DCs), thereby reducing the Th1-promoting capacity of DCs and shifting T cell responses towards Th2 [11]. Furthermore, PGE₂ promotes the induction of regulatory T cells via the induction of FoxP3 expression and the stimulation of transforming growth factor (TGF)- β secretion [12]. In the intestine, COX-2 is constitutively expressed by stromal cells [13] and prostaglandins are key factors in the physiological regulation of homeostasis and induction of oral tolerance to dietary antigens [14]. Another important contribution of COX-2-derived PGE₂ in the intestine is the maintenance of intestinal epithelial integrity [15]. Recent studies in experimental murine colitis have shown that PGE₂ is involved in the repair of mucosal injury via the induction of epidermal growth factor receptor on epithelial cells [16].

Hence, whereas COX-inhibiting drugs are used to limit inflammation and relieve pain, they may also disturb mucosal immunoregulatory processes and intestinal epithelial integrity, resulting in the stimulation of inflammatory responses. Ultimately, this can contribute to the development of intestinal disorders such as food allergy or inflammatory bowel disease.

In this study, we investigated the effect of a classic NSAID, diclofenac, on peanut-induced allergic sensitization and peanut-specific oral tolerance induction using mouse models. The Results show that oral administration of diclofenac is able to enhance the allergic response towards peanut extract (PE) in the presence of a mucosal adjuvant (cholera toxin, CT). Whether the effect of diclofenac is solely the result of COX inhibition or is also related to the disturbance of mucosal barrier function remains to be elucidated.

Methods

Mice and reagents

Five-week-old specific pathogen-free female C3H/HeOJCrI mice were purchased from Charles River (Lyon, France). All mice were maintained under barrier conditions in filter-topped macrolon cages with wood-chip bedding, at a mean temperature of $23 \pm 2^\circ\text{C}$, 50–55% relative humidity and a 12 h light/dark cycle. Acidified drinking water and laboratory food pellets were provided *ad libitum*. The experiments were approved by the Animal

Experiments Committee of the Utrecht University. Peanuts from the Golden Peanut plant (Alpharetta, GA, USA) were kindly provided by Intersnack Nederland BV (Doetinchem, the Netherlands) and PE was prepared as described previously [17]. CT was purchased from List Biological Laboratories Inc. (Campbell, CA, USA). Chemicals were obtained from Sigma Aldrich (Zwijndrecht, the Netherlands), unless stated otherwise.

Treatment protocols

Oral sensitization towards peanut. Mice ($n = 8$) were exposed by an intragastric gavage to 6 mg PE mixed with or without 15 μg CT for 3 consecutive days, and the dosage was repeated every week for 3 weeks (exposure on day 0, 1, 2, 7, 14 and 21). The control groups received phosphate-buffered saline (PBS) plus CT. Diclofenac (0, 1, 10 or 25 mg/kg, dissolved in sterile water (Braun, Melsungen, Germany) was administered via an intragastric gavage 2 h before PE+CT sensitization on days 0, 1 and 2. All mice received a challenge of 12 mg PE on day 26, and were sacrificed 1 day later (Fig. 1a). In order to investigate whether diclofenac induced gastrointestinal damage, groups of mice ($n = 4$) were exposed to diclofenac and PE+CT for 3 consecutive days as described for oral sensitization and were sacrificed 1 day later (Fig. 1a). The effect of diclofenac on COX mRNA expression in the intestine was determined 2 h after a single exposure to diclofenac.

Induction of tolerance towards peanut. Mice ($n = 8$) received 1 mg PE or PBS via an intragastric gavage for 3 consecutive days in order to induce oral tolerance. Diclofenac (1 mg/kg) was administered orally 2 h before tolerance induction. Immunization was performed intraperitoneally (i.p.) with 100 μg PE and alum (Imject, Thermo Scientific, Rockford, IL, USA) 21 and 28 days after the initial exposure, and mice were sacrificed on day 37 (Fig. 1b).

Measurement of peanut extract-specific immunoglobulins G1, G2a, E and of noexpmMCP-1

PE-specific antibodies in the serum were detected by ELISA as described previously [17]. mMCP-1 was determined using an ELISA kit (Moredun Scientific Ltd., Midlothian, Scotland) and performed according to the instructions of the manufacturer.

Tissue staining

Acetone-fixed cryostat sections of the small intestine were incubated with anti COX-2 polyclonal Ab (Cayman, Ann Arbor, MI, USA) diluted in PBS, 1% bovine serum

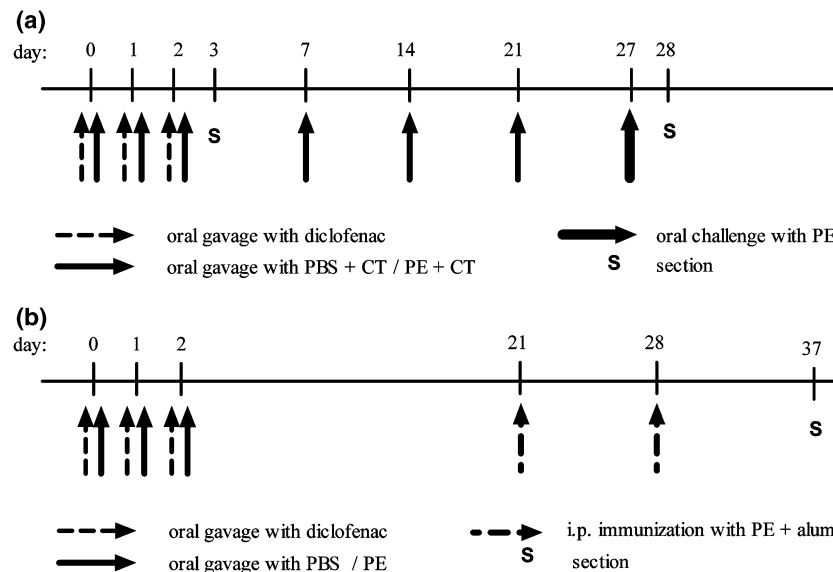


Fig. 1. Protocols to induce allergic sensitization (a) or oral tolerance (b) to peanut extract (PE) in mice.

albumin. Antibody specificity was checked using a COX-2 blocking peptide (Cayman) according to the instructions of the manufacturer. Staining was visualized by applying biotinylated swine anti-rabbit F(ab')₂ fragments (DAKO, Denmark) in PBS, supplemented with 5% normal mouse serum, followed by incubation with horseradish peroxidase-labelled streptavidin (DAKO) and aminoethylcarbazole as a substrate. Sections were counterstained with Mayer's haematoxylin (DAKO) and mounted with DAKO Faramount Aqueous mounting medium. To determine gastrointestinal damage, formalin-fixed paraffin sections of the stomach and acetone-fixed cryosections of the small intestine were stained with haematoxylin-eosin. To visualize the mucus layer, acetone-fixed cryosections of the small intestine were stained with Periodic Acid Schiff (PAS), followed by staining with Alcian Blue. Images were acquired using an Olympus BX60 microscope with a colorview III digital camera and Cell imaging software.

Measurement of prostaglandinE₂ in the intestine

A sample of the duodenum (without Peyer's patches) of 50–60 mg was washed three times in an ice-cold medium [RPMI-1640 supplemented with 100 IU/mL penicillin/streptomycin (pen/strep, Invitrogen, Breda, the Netherlands) and 10% fetal calf serum (FCS) (Greiner Bio-one, Alphen a/d Rijn, the Netherlands)] and incubated in 0.5 mL of the medium for 24 h at 37 °C, 5% CO₂. The supernatant was collected and stored at –20 °C until analysis. The levels of PGE₂ in the culture supernatant were determined using a commercially available ELISA (R&D Systems, Abingdon, Oxon, UK) according to the instructions of the manufacturer. The levels were normal-

ized for the exact weight of intestinal tissue used in the culture.

Lymphocyte culture and cytokine measurement

Spleen and mesenteric lymph node (MLN) single-cell suspensions (3.75×10^5 per well) were incubated in 200 μ L RPMI-1640 supplemented with 100 IU/mL pen/strep and 10% FCS in the presence or absence of 100 μ g/mL PE in 96-well plates for 96 h at 37 °C, 5% CO₂. Culture supernatants were harvested and stored at –20 °C until analysis.

In the culture supernatants, the levels of IFN- γ , IL-4, IL-5, IL-13 and IL-10 were determined using commercially available sandwich ELISA (IL-10 was obtained from BD-Pharmingen, Erembodegem, Belgium, and others from e-Bioscience, Halle-Zoersel, Belgium), according to the instructions of the manufacturers.

Epithelial cell culture, nuclear factor κ B and macrophage inflammatory protein-2 measurement

The murine small intestinal cell line m-ICc12, stably transfected with a nuclear factor- κ B (NF- κ B) luciferase reporter construct, was a kind gift from Dr A. Vandewalle (INSERM, Paris, France [18, 19]). m-ICc12 cells were cultured in DMEM/F12 (1:1 v/v), supplemented with pen/strep, 60 nM selenium, 5 g/mL bovine transferrin, 2 mM glutamine, 5 g/mL bovine insulin, 2 g/L glucose, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/mL epidermal growth factor, 2% FCS, 50 μ g/mL hygromycin and 15 mM HEPES. Cells were grown in a humidified incubator at 37 °C and 5% CO₂.

To assess activation, m-ICc12 cells were grown to confluence in 96-well plates (Greiner Bio-one) and subsequently incubated for 4 h with different amounts of diclofenac in the absence or presence of CT (20 µg/mL) or lipopolysaccharide (LPS, 10 ng/mL; O111:B4). The culture supernatant was harvested and stored at -20°C until the determination of macrophage inflammatory protein-2 (MIP-2). Cells were washed once with PBS, and lysed with 30 µL lysis buffer (Promega, Madison, WI, USA). Cell lysates were stored at -80°C or luciferase activity was measured immediately by adding a luciferase-assay mix (containing 20 mM tricine, 1.07 mM magnesium carbonate hydroxide, 2.67 mM magnesium sulphate, 0.1 mM EDTA, 33.3 mM dithiothreitol, 261 µM coenzyme A, 470 µM luciferin and 530 µM ATP) to a 20 µL sample. Luciferase activity was recorded using a luminometer (LUMIstar OPTIMA, BMG Labtech, Offenburg, Germany). MIP-2 ELISA (KOMA Biotech., Seoul, Korea) was performed according the instructions of the manufacturer.

Real time-polymerase chain reaction for cyclo-oxygenase-2 messenger RNA expression

mICc12 cells were grown to confluence in six-well plates (Greiner Bio-one) and subsequently incubated for 4 h with different amounts of diclofenac in the absence or presence of CT (20 µg/mL). Cell were washed with PBS, lysed with RNA Instapure[®] (Eurogentec, Maastricht, the Netherlands) and stored at -80°C until the isolation of RNA. Approximately, 50 mg of the small intestine (without Peyer's Patches, PP) and 2 PP were dissected and stored at -80°C until further use. The tissue was homogenized in 1 mL RNA Instapure[®] using a Mixer-Mill (Qiagen, Venlo, the Netherlands). RNA was extracted from the RNA Instapure[®] samples using chloroform and isopropanol. The RNA content was quantified by measuring the optical density at 260 nm, and RNA integrity was confirmed by measuring A_{260}/A_{280} on a SmartSpecPlus (Bio-Rad Laboratories, Veenendaal, the Netherlands). Subsequently, RNA was transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the

manufacturer's protocol. Real-time PCR was performed using an iCycler iQ system (Bio-Rad Laboratories), and amplification was performed using IQ[™] SYBR Green[®] Supermix (Bio-Rad Laboratories) with a 0.4 µM final primer concentration. The primers used were: COX-2 sense 5'-TGC AAG ATC CAC AGC CTA CC-3', antisense 5'-GCT CAG TTG AAC GCC TTT TG-3'; β -actin sense 5'-ATG CTC CCC GGG CTG TAT-3', antisense 5'-CAT AGG AGT CCT TCT GAC CAT TC-3'. For each sample, mRNA expression was normalized for the detected C_t value of β -actin.

Statistics

Data are presented as the group mean $\pm$ SEM and analysed using GraphPad Prism (La Jolla, CA, USA) software. One-way ANOVA was performed, followed by Bonferroni as a *post hoc* test. Before testing, logarithmic transformation was performed on antibody and cytokine data from *in vivo* studies to achieve a normal distribution. Differences were considered significant when *P*-values were <0.05 .

Results

Administration of diclofenac before peanut sensitization resulted in enhanced peanut-specific responses

Diclofenac were administered in different doses (0, 1, 10 and 25 mg/kg) before allergic sensitization to determine its influence on peanut-specific responses. Exposure to PE+CT induced PE-specific IgG1, IgG2a and IgE in the serum, detected after 28 days (Fig. 2). Administration of diclofenac before PE+CT exposure resulted in significant increases of serum levels of PE-specific IgG1 and IgE (Fig. 2). Furthermore, mMCP-1, a measure for mast cell degranulation *in vivo*, was enhanced by diclofenac upon an oral challenge with PE (Fig. 3).

When mice were treated with 0 or 25 mg/kg diclofenac and PE in the absence of CT, no PE-specific antibodies could be detected (data not shown), indicating that diclofenac at this dose is not able to elicit an allergic response in the absence of the adjuvant CT.

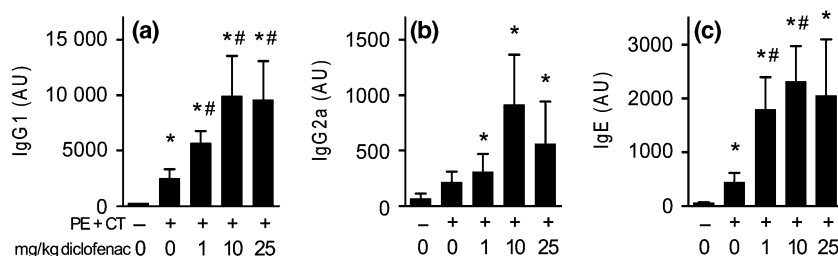


Fig. 2. Effect of diclofenac on peanut extract (PE)-specific antibody production. Mice ($n=8$) were treated with indicated amounts of diclofenac, and sensitized to PE. After 4 weeks, PE-specific IgG1 (a), IgG2a (b) and IgE (c) in the serum were determined. Data are presented as mean $\pm$ SEM. * $P<0.05$ compared with no peanut sensitization, # $P<0.05$ compared with no diclofenac exposure.

Administration of diclofenac resulted in enhanced T cell responses in peanut sensitization

To investigate the effect of diclofenac on PE-specific T cell responses, splenocytes from treated animals were cultured for 96 h in the presence or absence of PE. Exposure to PE+CT resulted in detectable levels of IFN- γ , IL-13 and IL-10 in the culture supernatant (Fig. 4). Administration of diclofenac significantly increased the levels of IFN- γ and IL-13, but not IL-10 (Fig. 4). The levels of IL-4 and IL-5 were below the detection limit. When mice were treated with PE alone (in the absence of CT), no cytokine production could be found.

Tolerance induction to peanut extract was only slightly altered upon diclofenac exposure

Next, the effect of diclofenac on the induction of oral tolerance to PE was determined. Mice were orally tolerized by the administration of a low dose of PE (1 mg) for 3

consecutive days and 1 mg/kg diclofenac was administered 2 h before each oral PE exposure. Mice were immunized 3 weeks later via two i.p. injections with PE/alum at a 1-week interval (Fig. 1). As expected, oral PE exposure before immunization resulted in lower PE-specific IgG1 and IgE in the serum (Fig. 5) and the cytokine production by splenocytes after *ex vivo* PE restimulation was diminished (Fig. 6), demonstrating the induction of oral tolerance.

Administration of diclofenac before oral exposure to PE did not abrogate oral tolerance induction, as PE-specific antibodies in serum were comparably low in diclofenac-treated and vehicle-treated tolerized mice (Fig. 5). In addition, cytokine levels in the supernatant of cultured splenocytes from PE-tolerized, diclofenac-treated mice were significantly lower compared with those in splenocyte cultures of non-tolerized mice. Noticeably, the *ex vivo* PE-specific cytokine levels of diclofenac-treated tolerized mice were significantly higher compared with vehicle-treated tolerized mice, except in the case of IL-4 (Fig. 6d). Cytokine levels in the non-tolerized groups were comparable.

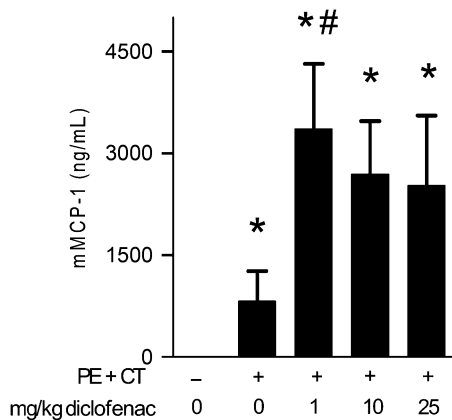


Fig. 3. Effect of diclofenac on mMCP-1 release after an oral challenge. Indicated amounts of diclofenac were administered before peanut extract (PE) sensitization. mMCP-1 was measured in the serum from blood samples taken 30 min after an oral PE challenge. Data are mean $\pm$ SEM from eight mice per treatment group. * P < 0.05 compared with no-PE sensitization, # P < 0.05 compared with no diclofenac exposure.

Exposure to diclofenac did not alter cyclo-oxygenase-2 expression but resulted in changes in the gastrointestinal morphology

COX-2 expression in the small intestine was assessed in intestinal tissue sections 2 h after diclofenac exposure. Although diclofenac is known to inhibit the activity of COX enzymes, the expression of COX-2 in the small intestine was not diminished as shown by immunohistochemistry (Fig. 7a). These findings were confirmed by RNA analysis of COX-2 in the intestine (data not shown). Assessment of PGE₂ in the duodenum 24 h after exposure to diclofenac and PE+CT for 3 consecutive days also revealed no significant changes in the PGE₂ levels (Fig. 7d). At this time-point, the gastro-intestinal morphology, however, revealed disturbances in the small intestine. The epithelial cells had a more flattened appearance upon exposure to diclofenac (Fig. 7b), but there was no influx of neutrophils in the tissue. The morphology of the

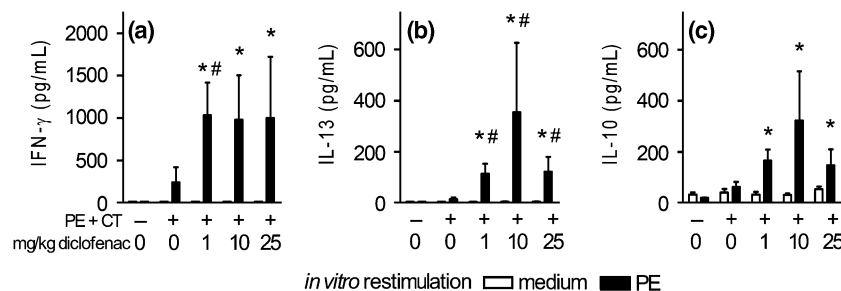


Fig. 4. Effect of diclofenac on cytokine production in the spleen. Mice (n = 8) were treated with indicated amounts of diclofenac and sensitized to peanut extract (PE). Splenocytes were stimulated *ex vivo* and the levels of IFN- γ (a), IL-13 (b) and IL-10 (c) were determined in the culture supernatant. Data are depicted as mean $\pm$ SEM. * P < 0.05 compared with no PE sensitization, # P < 0.05 compared with no diclofenac exposure.

stomach showed erosions in the epithelium (Fig. 7c) that were present in three of four mice within the 10 and 25 mg/kg diclofenac-exposed groups. The erosions in the stomach were accompanied by an influx of neutrophils. Stomach ulcers were absent in all animals. Exposure to diclofenac alone did not result in changes in the morphology or in a detectable influx of neutrophils in the small

intestine within 2 h after a single exposure. In addition, no changes in the intestinal mucus layer (examined with PAS-Alcian Blue staining) could be observed (data not shown).

Diclofenac did not alter cholera toxin-mediated nuclear factor κ B activation in epithelial cells in vitro

In addition, we investigated whether diclofenac could directly modulate epithelial cell responses. Mouse intestinal epithelial cells (IEC) (m-ICc12), stably transfected with an NF- κ B-luciferase reporter construct, were incubated with various concentrations of diclofenac for 4 h in the presence or absence of CT or LPS. NF- κ B activation and MIP-2 production were analysed after 4 h of stimulation. Both CT and LPS were able to stimulate m-ICc12 cells to activate NF- κ B and to release MIP-2 (Figs 8a–b). Diclofenac was not able to change CT- or LPS-induced NF- κ B activation or MIP-2 release (Figs 8a–b). Conversely, COX-2 mRNA expression was significantly inhibited *in vitro* upon diclofenac exposure (Fig. 8c).

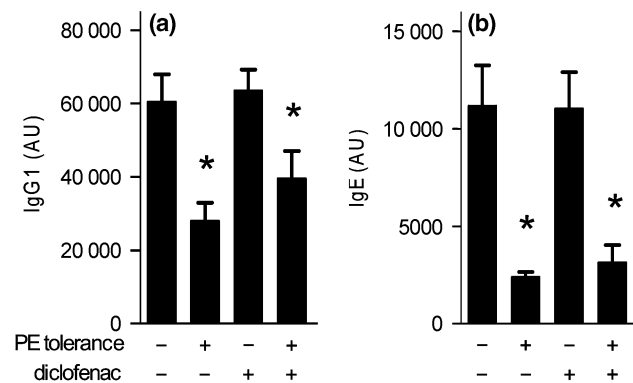


Fig. 5. Effect of diclofenac on peanut extract (PE)-specific antibodies after oral tolerance induction. Mice ($n = 8$) were treated with diclofenac (1 mg/kg) before oral exposure to phosphate-buffered saline (PBS) or PE. Three weeks later, they were immunized intraperitoneally (i.p.) with PE + alum. PE-specific IgG1 (a) and IgE (b) were determined in the serum. Data are presented as mean $\pm$ SEM. * $P < 0.05$ compared with the matched control (pre-treatment with PBS).

Discussion

NSAIDs and especially aspirin are linked to exacerbations of urticaria and asthma, although part of this effect may be related to hypersensitivity to the drug itself [20, 21]. Evidence in humans of enhancement of allergic responses

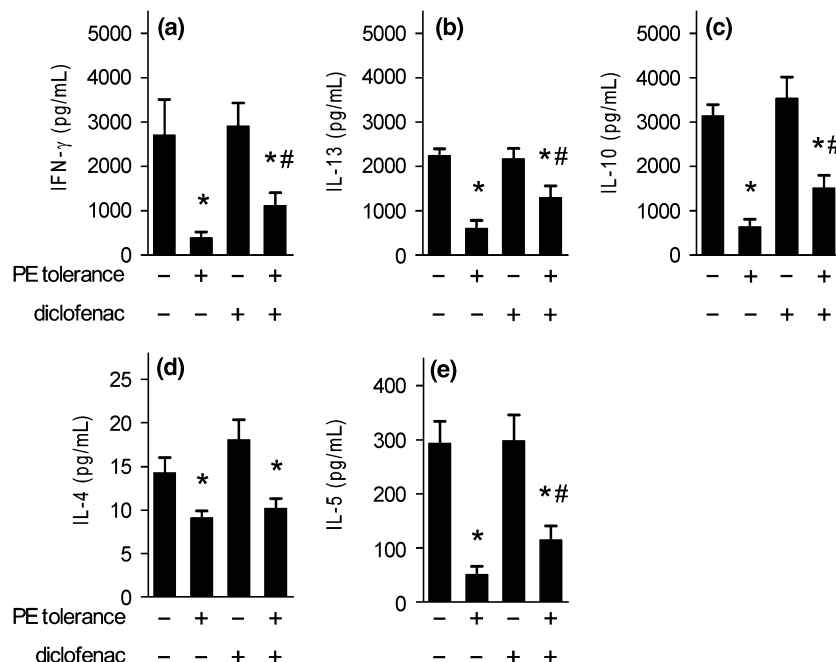


Fig. 6. Effect of diclofenac on cytokine levels in the spleen after oral tolerance induction. Mice ($n = 8$) were treated with diclofenac (1 mg/kg) before oral exposure to PBS or PE. Three weeks later, they were immunized intraperitoneally (i.p.) with PE + alum. Splenocytes were stimulated *ex vivo* and the levels of IFN- γ (a), IL-13 (b), IL-10 (c), IL-4 (d) and IL-5 (e) were determined in the culture supernatant. Data are presented as mean $\pm$ SEM. * $P < 0.05$ compared with the matched control (pre-treatment with PBS), # $P < 0.05$ compared with no diclofenac exposure.

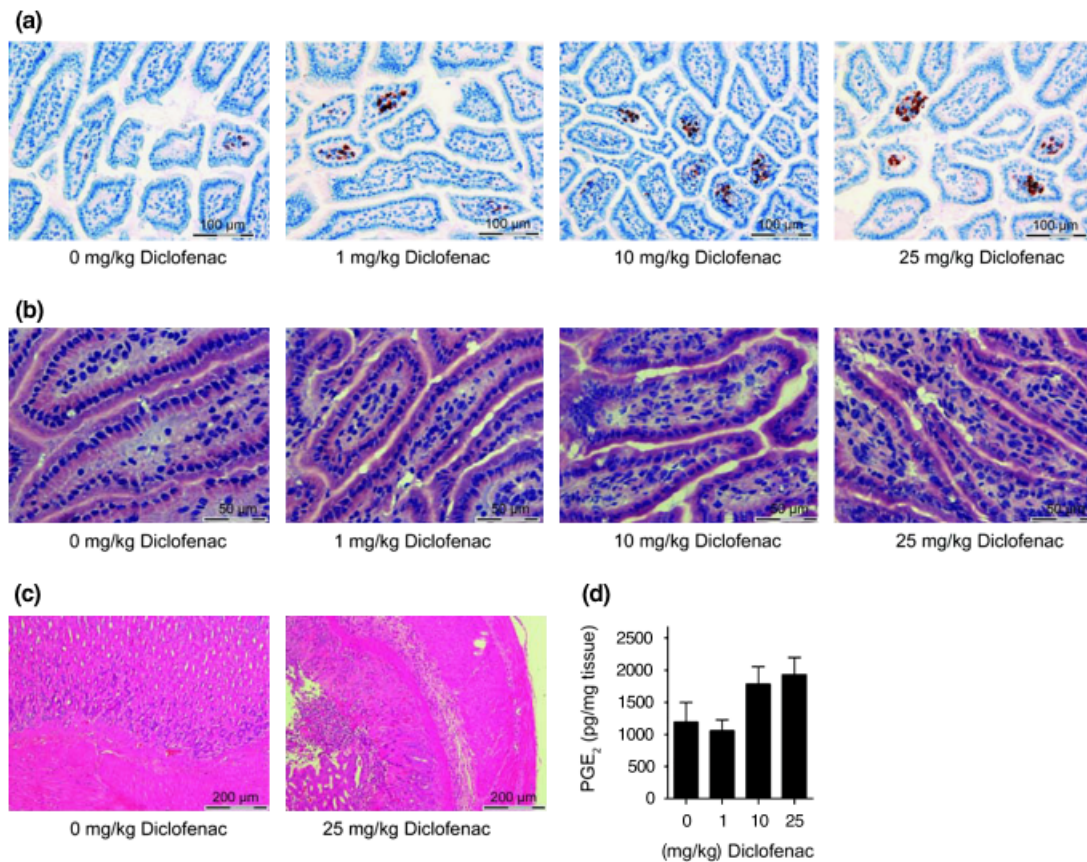


Fig. 7. Cyclo-oxygenase-2 (COX-2) expression and gastrointestinal damage upon diclofenac exposure. (a) Mice ($n = 3$) were treated orally with 0, 1, 10 or 25 mg/kg diclofenac and the small intestine was dissected 2 h later. Cryosections of the small intestine were stained in red for COX-2 (representative images are shown). (b)–(d) Mice ($n = 4$) were orally exposed to 0, 1, 10 or 25 mg/kg diclofenac, followed by PE+CT for 3 consecutive days. The small intestine and the stomach were dissected 24 h after the last exposure. (b) Representative images of haematoxylin–eosin (H&E)-stained cryosections of the small intestine. (c) Representative example of epithelial erosion in H&E-stained paraffin sections of the stomach. (d) PGE₂ in the supernatant of cultured intestinal samples were determined using ELISA, and normalized for sample weight.

by NSAIDs comprises mainly of an exercise-induced anaphylaxis in combination with aspirin [22]. One case study has observed a synergistic effect of NSAIDs in inducing anaphylaxis in a shellfish-allergic individual with antigen-specific IgE [23].

The current study shows that diclofenac-enhanced allergic responses to PE, but did not elicit an allergic response in the absence of CT. In the present peanut allergy model, CT is used as a tool to study allergic sensitization. Among other actions, CT alters the normally tolerogenic environment of the intestine through enhancement of antigen uptake by DCs [24]. Furthermore, it has been shown that CT directly stimulates DC by up-regulating Th2 driving co-stimulatory molecules [25]. Enterotoxins such as enterotoxin B from *Staphylococcus aureus* (SEB) and thermolabile enterotoxin from *Escherichia coli* (LT) have comparable adjuvant capacities in mouse models for allergic sensitization [1, 26] and exert comparable effects on DCs [27]. We conclude that diclofenac and possibly other NSAIDs should be considered a risk

factor for the induction of allergic responses when other circumstances (i.e. gastrointestinal infections, genetic susceptibility) are also in favour of allergy induction.

Treatment with diclofenac will result in the inhibition of COX activity and hence in the modulated production of prostaglandins, with multiple effects. First, prostaglandins may be involved in the induction of oral tolerance via an IL-4-dependent mechanism [6]. However, in the present study, the low levels of antibody production in the case of oral tolerance were not reversed by diclofenac, although diclofenac increased the low T cell responses in the tolerance model. To test the induction of oral tolerance, a peripheral challenge was performed by means of two i.p. injections with PE+alum, which results in a different magnitude of antibody responses compared with oral sensitization with CT. This may clarify the absence of an effect on antibody responses when using a low dose of diclofenac. As the alterations in T cell responses are indicative of an effect on tolerance, it may be worthwhile to investigate higher diclofenac doses or more continuous

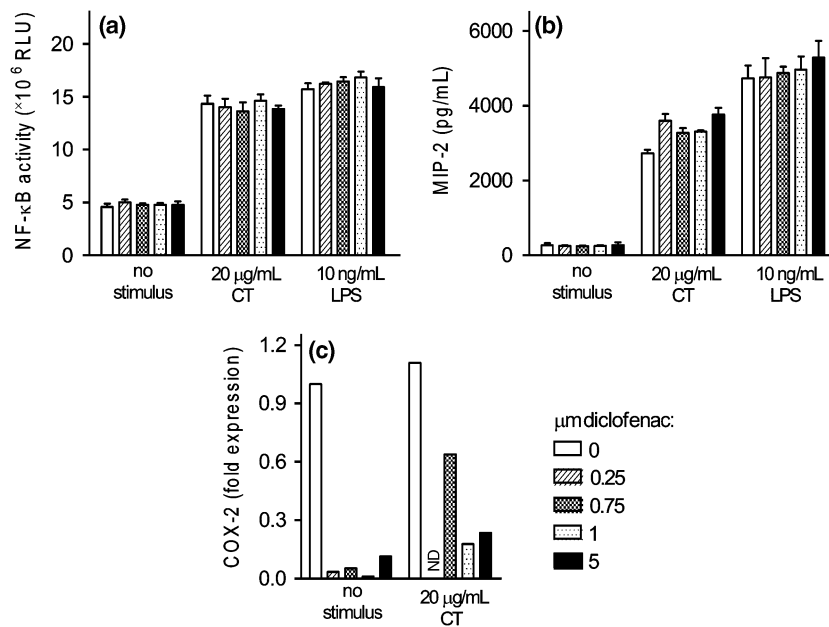


Fig. 8. Effect of diclofenac on cholera toxin (CT)- and lipopolysaccharide (LPS)-induced activation of mICc12 cells. mICc12 cells were grown to confluence, exposed to 0, 0.25, 0.5, 1 and 5 μM diclofenac and incubated for 4 h with 20 $\mu\text{g/mL}$ CT or 10 ng/mL lipopolysaccharide (LPS). (a) Nuclear factor- κB (NF- κB) activation is measured using a luciferase reporter construct and expressed as relative light units. (b) MIP-2 is measured in the culture supernatant using ELISA. Data are presented as mean $\pm$ SD from a triplicate from one representative experiment out of three. (c) Cyclo-oxygenase-2 (COX-2) mRNA content, determined by RT-PCR and expressed as fold expression, after normalization for β -actin; ND, not determined.

exposure to modulate the antibody responses in oral tolerance induction.

Secondly, as prostaglandins and related leukotrienes are involved in histamine release from mast cells, a direct effect of diclofenac and other NSAIDs on this process is also conceivable, although contradicting results have been obtained [28]. In general, NSAIDs are able to inhibit histamine release, but at much higher concentrations than needed to inhibit COX activity. Moreover, a direct effect on mast cell release does not explain the observed increase in PE-specific antibodies and T cell responses as observed in the present study.

Third, and probably most relevant to the present study, prostaglandins have been shown to be important in maintaining mucosal homeostasis, and the repair of mucosal damage involves the presence of PGE_2 [15, 16]. In this context, it is of importance that the use of conventional NSAIDs, like diclofenac, is accompanied by a high incidence of mucosal damage, including gastric erosions, ulcers and perforation and bleeding of the gastroduodenal tract [29, 30]. Reduced levels of PGE_2 in the intestine as a consequence of COX-inhibition may worsen the outcome due to the absence of appropriate tissue repair (after CT-induced damage). We did not observe decreased expression of COX-2 in the small intestine 2 h after diclofenac exposure in the present study. This is in line with results from others showing that NSAIDs inhibit COX-2 activity without affecting mRNA and protein expression in the colon [31]. However, we did

not find a significant change in the levels of PGE_2 in the intestine after diclofenac exposure. This time-point is probably beyond the pharmacological efficacy window of the drug, although it is consistent with the absence of an effect on COX expression. Nevertheless, the absence of diclofenac-mediated inhibition of COX-2 expression in the intestine is in contrast with our *in vitro* data in IEC. Possibly, the constitutive expression of COX-2 in stromal cells of the intestine [13] may also count for the difference between the present *in vivo* and *in vitro* results.

Diclofenac-induced abrogation of the protective mucus layer may result in increased accessibility of the IEC for diclofenac itself, and also of luminal contents such as bacterial products and food antigens [32]. The treatment of IEC with diclofenac *in vitro* resulted in profound inhibition of COX-2, but did not result in the activation of NF- κB . In addition, diclofenac was not able to enhance CT- or LPS-induced NF- κB activation in these cells. Therefore, a direct stimulating effect of diclofenac on IEC *in vivo* resulting in induction of a mucosal immune response appears to be unlikely. The lack of induction of allergic responses by diclofenac in the absence of CT, as found in the present study, also indicates that diclofenac itself is unable to induce a mucosal immune response. This is in line with the recent findings of Riemer *et al.* [33] showing that disturbance of gastric mucosa had to precede an immune response to diclofenac. In contrast, it has been observed that oral dosing of diclofenac is able to elicit an immune response to an i.p.-administered antigen

[7] and to high doses of orally administered antigen (I. Ludwig, unpublished data). This increased response consisted of induction of antigen-specific IgG1 and IgG2a antibodies, and increased levels of IFN- γ in the culture supernatant of *ex vivo* restimulated spleen cells. These data suggest that (the route of) antigen entrance (peripheral or mucosal) together with the antigen concentration is decisive for the adjuvant capacity of diclofenac, although the mechanism behind these differences remains to be elucidated.

The present study showed gastrointestinal damage after exposure to diclofenac and PE+CT for 3 days. It is not clear whether damage to the stomach or damage to the small intestine contributed equally to the observed increase in allergic symptoms after diclofenac exposure. Notably, diclofenac-induced enteropathy has been shown to result in an increased intestinal permeability [29, 32], and the effect of diclofenac on the allergic response may result from increased intestinal permeability of the antigen. This idea is supported by a recent study in which increased serum levels of dietary gliadin were found in healthy subjects after the application of aspirin, diclofenac or loxoprofen in representative clinical doses [34]. The combined exposure to diclofenac and CT probably resulted in a more severe epithelial damage than exposure to diclofenac alone. This would explain the absence of an effect of diclofenac on allergic sensitization in the absence of CT. Whether the *in vitro* antimicrobial properties of diclofenac as described previously [35] alter the microbial status of the intestine and contribute to the observed results remains unclear.

To evaluate the risk of using NSAIDs in allergy-susceptible individuals, it is of importance to investigate to what extent COX-2 inhibition or intestinal barrier disruption contributes to the modulation of PE responses by diclofenac and other NSAIDs.

In conclusion, our data show that the use of NSAIDs can contribute to the development of food-allergic responses. It is of importance to investigate whether diclofenac and other NSAIDs are indeed able to enhance existing peanut responses in humans as this would implicate an important risk factor for the use of these drugs in food-allergic individuals.

Acknowledgements

We thank Dr Raoul Kuiper for evaluation of the gastrointestinal morphology. I. L. is funded by TIPharma, Grant D3-201 TIP-ADR.

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