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An Animal Model for Wheat Allergy Skin Sensitisation: A Comparative Study in Naive versus Tolerant Brown Norway Rats

Anne-Sofie Ravn Ballegaard Charlotte Bernhard Madsen
 Katrine Lindholm Bøgh

National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark

Keywords

Food allergy · Gluten · Skin sensitisation · Animal model · Acid-hydrolysed gluten

Abstract

Background: Allergic sensitisation to foods may occur in infancy without prior oral exposure to the offending food, leading to the assumption that food allergy sensitisation may occur through the skin. Concerns have been raised regarding the safety of use of personal care products containing hydrolysed wheat proteins, since these products have been shown to induce allergy through the skin, and even cause an abrogation of an already established oral tolerance. **Objective:** To establish an animal model for food allergy skin sensitisation and compare the sensitising capacity of an unmodified and an acid-hydrolysed gluten product via slightly damaged skin in naïve versus tolerant rats. **Methods:** Gluten products were applied on the slightly damaged skin of naïve or tolerant Brown Norway (BN) rats without adjuvant 3 times per week for 3 or 5 consecutive weeks. The effect of the skin applications was evaluated by means of different ELISAs and immunoblotting. **Results:** A robust animal model was developed for food allergy skin sensitisation. In naïve rats, both gluten products were able to induce a statistically significant level of

specific antibodies and sensitise through the skin, but in the wheat-tolerant rats, only the acid-hydrolysed gluten was able to sensitise through the skin, albeit at a level much lower than in the naïve rats. Results showed that new epitopes had been developed as a result of acid hydrolysis but original epitopes were maintained. This may explain why only the acid-hydrolysed gluten could induce specific antibody responses in the tolerant animals. **Conclusions:** This study showed that it is possible to sensitise BN rats through slightly damaged skin, and that the sensitising capacity is heavily influenced by the tolerance status of their immune system and the degree of modification of the wheat products.

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Introduction

Immunoglobulin E (IgE)-mediated food allergy is the most common type of adverse reaction towards food proteins. It is associated with the presence of allergen-specific IgE that has emerged from a sensitisation phase followed by an elicitation phase, causing the allergic reaction

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[1]. The default immune response to dietary antigens in the gastrointestinal (GI) tract is active immune tolerance, namely oral tolerance [2]. Failure to develop, or an abrogation of, oral tolerance may cause sensitisation. Allergic reactions to foods have been reported to occur after the first known ingestion, which suggest sensitisation through routes other than the GI tract [3].

The skin is an alternative route of sensitisation. It is a unique organ that serves as a protective barrier between the host organism and its external environment. Minimising water loss from the body and preventing pathogens and allergens from entering the organism may be the skin's primary functions [4]. Subjects with disrupted barrier functions such as atopic dermatitis (AD) or a loss-of-function mutation in the filaggrin gene have an increased risk of developing a food allergy [5, 6]. This has been proposed, in particular, in relation to peanut allergy [7, 8]. Induction of food allergy due to skin exposure of food proteins is not fully understood; this makes it highly relevant to investigate.

Indications of food allergy sensitisation through the skin, together with an increased usage of natural materials and derivatives thereof in personal care products intended for skin application, have led to concerns about the safety of such products. Natural components found in personal care products may be proteins from some of the major allergenic foods such as cow's milk, peanut, soy, and wheat [8–10]. Wheat proteins, particularly, and their derivatives are used as components in various cosmetics and personal care products [11]. It is the quality of gluten that gives wheat its unique capabilities, including the capacity for water absorption, viscosity, and elasticity [12]. Gluten contains hundreds of proteins present either as monomers, oligomers, or polymers [13].

By definition, gluten proteins are not soluble in water. Hydrolysis with enzymes or acid can alter their structure and size and result in soluble protein hydrolysates. The procedure and degree of hydrolysis both depend on the desired function and the manufacturer. Treatment with acid can, furthermore, result in partial deamidation of the proteins. Chemical deamidation of gluten removes the amide from glutamine or asparagine, forming the corresponding carboxylic acid, glutamate or aspartate, together with free ammonia. This process changes the potential charge and thus increases the solubility of gluten [14, 15]. Acid hydrolysis of gluten induces emulsifying properties which makes it useful in different kinds of food products, but also in personal care products for the hair and body [16]. In different parts of the world, hydrolysed wheat proteins have been reported to cause

food-allergic reactions, even anaphylaxis, in patients tolerant to wheat [17–20]. This includes cases of allergic skin reactions after applying personal care products containing hydrolysed wheat proteins [16, 17, 21]. Especially in Japan, allergic reactions to wheat products have been caused by facial soaps containing acid-hydrolysed wheat proteins. The allergic reactions were skin symptoms and wheat-dependent exercise-induced anaphylaxis (WDEIA) [17, 22]. Acid hydrolysis of gluten may change the epitope patterns and thereby influence the response of the immune system. The ability of acid-hydrolysed gluten to cause a *de novo* sensitisation and the possibility of breaking an already established oral tolerance to unmodified wheat proteins have increased the concerns about the safety of use of these proteins in personal care products.

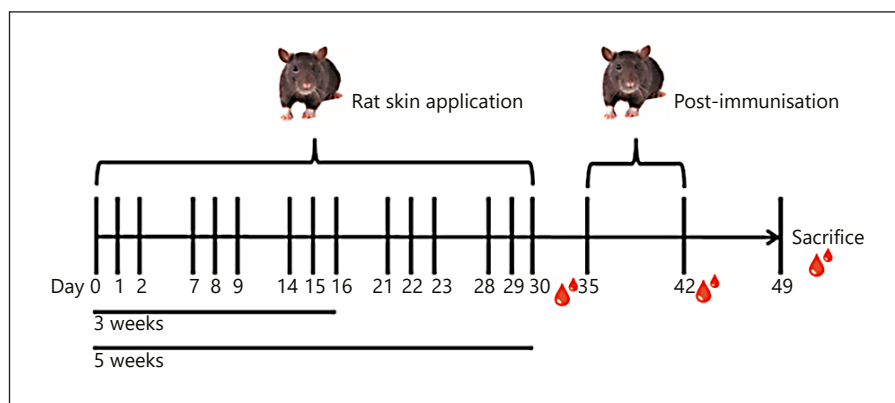
Animal models may contribute to the understanding of the role of protein sensitisation through the skin. It is difficult to study the sensitisation phase in humans since we are exposed to an uncontrolled variety of food and inhalant allergens over time and also due to ethical reasons. Therefore, it is justified to use animal models, where allergen exposure can be controlled, and enabling extensive investigation within a well-defined genetic background. Different animal models have shown that sensitisation through the skin can occur [23–26]. Allergens have been applied on intact skin or on mechanically disrupted skin caused by tape-stripping [24, 25, 27]. Different food allergens have been used in the animal models such as peanut, hazelnut, and cashew nut protein extracts, and hen's egg ovalbumin (OVA), resulting in elevated levels of antigen-specific IgE [24–27]. Few animal studies have investigated allergic reactions towards hydrolysed wheat products [28–30]. In this study, the aim was to establish an animal model for food allergy skin sensitisation and investigate the sensitising capacity of an unmodified and an acid-hydrolysed gluten product in naïve versus tolerant Brown Norway (BN) rats.

Material and Methods

Gluten Products

The 2 different gluten products, i.e., unmodified and acid-hydrolysed gluten, were kindly provided by Tereos Syral (Aalst, Belgium). They were dissolved in sterile PBS (137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄×2H₂O, and 1 mM KH₂PO₄ in Milli Q water, pH 7.2) before use. Endotoxin content of both products were shown to be <10 EU/mg protein with the Pierce™ LAL chromogenic endotoxin quantification kit (ThermoFisher Scientific, MA, USA) according to the manufacturer's instructions.

Fig. 1. Animal experimental design. Groups of 6 Brown Norway rats were dosed on the skin by application with PBS (control), unmodified gluten, or acid-hydrolysed gluten for 1 h/day for 3 consecutive days for 3 or 5 weeks. Subsequently, 2 post-immunisations were given with a 1-week interval. Blood samples were collected throughout the study. Rats were sacrificed on day 49 (photos: Colourbox.com).



Animals

BN rats from the in-house breeding colonies at the National Food Institute, Technical University of Denmark, were used. Rats were raised and bred on either (1) an in-house diet free from wheat [31] for >3 generations to ensure immunological naïve animals with respect to wheat proteins, or (2) conventional rat chow containing wheat (Altromin 1314, with a protein content of 6.73% originating from gluten, Altromin, Lage, Germany). Animals of both genders aged 5–8 weeks were used. Animals were housed in macrolon cages ($n = 3/\text{cage}$) at $22 \pm 1^\circ\text{C}$ with a relative humidity of $55 \pm 5\%$. Air was changed 8–10 times/h and electric lights were on from 9.00 a.m. to 9.00 p.m. Diet and acidified water were given ad libitum. The animals were inspected twice a day and body weights were recorded every week. At the end of the studies, all animals were scarified by exsanguination using carbon dioxide as anaesthesia. Blood was collected and converted into sera and stored at -20°C until analysis.

Animal Studies

Pilot Studies: Skin Histology and Water Evaporation

In all pilot studies, rats were bred and raised on a diet free from wheat. A first step in developing an animal model is to have a reliable and reproducible method for the application of the products used for sensitisation, where a control of the condition of the skin can be performed.

Pilot Experiment 1

Different methods for hair removal on the abdomen were tested: an electric shaver, a razor, and 3 different depilatory creams (Silkia, Lino Care Ltd., Manchester, UK; pharmacy depilatory cream, Glostrup Pharmacy, Glostrup, Denmark; and Veet, Reckitt Benckiser, Slough, UK). The creams were used according to the manufacturers' instructions. After hair removal, the animals were tape-stripped 10 times with cellophane tape. They were sacrificed immediately after hair removal or tape-stripping, and the skin was subsequently excised, embedded in paraffin, cut, and stained with haematoxylin and eosin (HE) for visualisation of the skin layers.

We decided to remove hair with an electric shaver, as this was efficient and did not damage the skin.

Pilot Experiment 2

From pilot experiment 1, we knew that shaving with or without tape-stripping left the epidermis intact. In order to have 1 model

with intact skin and 1 with slightly damaged skin, we introduced gentle scratching with sandpaper (grit 400). We also wanted to investigate the effect (histology and water evaporation) on the skin of repeating the procedures over several weeks.

Twelve animals were allocated into 6 groups. All animals were shaved on the abdomen, scratched, and treated within an area of approximately $1 \times 1 \text{ cm}^2$. One animal in each group was dosed with PBS and the other with acid-hydrolysed gluten. Dosing and the dosing regimen were as described in the next section: "Establishment of a Rat Skin Sensitisation Model." The first 2 animals were sacrificed immediately after the first day of dosing. Subsequently, 2 animals were sacrificed 2 days after the first, second, third, fourth, or fifth dosing period, respectively. The skin was harvested and stained with HE.

For further examination of the skin condition due to the skin treatment and product application, water evaporation was measured from the area of skin application. Twelve animals were allocated into 2 groups, 1 group dosed with PBS and 1 group dosed with acid-hydrolysed gluten. All animals were shaved on the abdomen and scratched as described above for the histology study, and then dosed as described below. All 12 rats were sacrificed after 2 weeks of skin application. Water evaporation was measured 3 times for approximately 20 s for each step of the skin application, before and after shaving, after scratching, and after application for 1 h. Water evaporation was measured with a Tewameter[®] TM 300 (Courage+Khazaka Electronic GmbH, Cologne, Germany). Results from water evaporation measurements are expressed as the level of trans-epidermal water loss (TEWL).

Establishment of a Rat Skin Sensitisation Model

Rats included in this study were bred and raised on a diet free from wheat. Rats were shaved on the abdomen with an electric shaver (Oster, PowerPro Ultra, blade 50). Every week the skin was slightly damaged by scratching using sandpaper (grit 400). After the pretreatment, 100 μL of PBS, or 100 μL of PBS with 50 or 500 μg unmodified gluten or acid-hydrolysed gluten was applied on a $1 \times 1 \text{ cm}^2$ area without the use of adjuvant. To avoid oral exposure, the dosed skin was covered with an elastic gauze bandage wrapped around the abdomen. Rats were placed alone in a cage for 1 h. Afterwards, the dosing area was rinsed with water and the rats were placed in their original cages. Products were applied on the skin for 1 h/day for 3 consecutive days, after which the rats rested for 4 days. This was repeated for either 3 or 5 weeks. After the skin ap-

plication, rats were post-immunised 2 times with a 1-week interval (Fig. 1). Rats were post-immunised either intraperitoneally (i.p., 50 µg in 0.5 mL sterile PBS) or by oral gavage (50 mg in 1 mL sterile PBS) with the same product as was applied on the skin. PBS-treated control rats were post-immunised with acid-hydrolysed gluten. Rats were sacrificed 1 week after the last post-immunisation, and blood was collected and stored at -20 °C until use.

Comparison of Naïve and Tolerant Rats

Rats were bred and raised either on a diet free from wheat or a conventional rat chow. This study was performed as described above with the following exceptions: rats were dosed with PBS, 500 µg unmodified gluten, or acid-hydrolysed gluten. Skin sensitisation was performed for 5 weeks followed by 2 post-immunisations given by oral gavage (50 mg in 1 mL sterile PBS).

Enzyme-Linked Immunosorbent Assay

Sera from skin sensitisation studies were analysed by various ELISAs for specific IgG₁, specific IgE, avidity, and the competitive capacity of the 2 products. For all ELISA experiments, all reagents and incubation periods were at room temperature (RT) in the dark on a shaking table for 1 h unless otherwise described. Between each step, the ELISA plates were washed 5 times in PBS with 0.01% (w/v) Tween 20 (PBS-T). Positive and negative serum control pools were included on each plate for indirect and antibody-capture ELISA. For development of the enzymatic reaction, plates were incubated for 12 min with 100 µL/well TMB ONE™ (4380A, Kementec Diagnostics, Taastrup, Denmark). The reaction was stopped with 100 µL/well 0.2 M sulfuric acid. Absorbance was measured at 450–630 nm. Detection limits were determined as the mean absorbance for the negative control serum plus 3 times the standard deviation (SD).

Detection of Specific IgG₁ by Indirect ELISA

For detection of unmodified and acid-hydrolysed gluten-specific IgG₁, 96-well Maxisorp plates (NUNC, Roskilde, Denmark) were coated overnight at 4 °C with 100 µL/well of 2 µg/mL unmodified or acid-hydrolysed gluten in carbonate buffer (15 mM Na₂CO₃ × 10 H₂O, 35 mM NaHCO₃, pH 9.6). Plates were incubated with 50 µL/well serial 2-fold diluted rat sera in PBS-T starting at 1:8. Subsequently, plates were incubated with 100 µL/well of secondary antibody, mouse anti-rat IgG₁ labelled with horseradish peroxidase (HRP, 3060-05, Southern Biotech, Birmingham, AL, USA) diluted 1:20,000 in PBS-T. Afterwards, plates were washed twice in tap water and developed. Values are expressed as log₂ titres with a cut-off of optical density (OD) 0.1.

Detection of Specific IgE by Antibody-Capture ELISA

For the detection of specific IgE, antibody-capture ELISA was performed. Maxisorp plates were coated overnight at 4 °C with 100 µL/well of 0.5 µg/mL mouse anti-rat IgE (HDMAB-123 HydriDomus, Nottingham, UK) in carbonate buffer (pH 9.6). After coating, plates were blocked for 1 h at 37 °C with 200 µL/well of 3% (v/v) rabbit serum (S2500, Almeco, Esbjerg, Denmark) in PBS-T for the detection of unmodified gluten-specific IgE or 3% (w/v) skimmed milk powder (SMP, 70166, Sigma-Aldrich, St. Louis, MO, USA) in PBS-T for the detection of acid-hydrolysed gluten-specific IgE. After blocking the plates, rat serum samples were added in serial 2-fold dilution starting at 1:8 in PBS-T. Subsequently, the plates were incubated with 50 µL/well of digoxigenin (DIG)-coupled un-

modified gluten diluted 1:500 in 3% (v/v) rabbit serum or DIG-coupled acid-hydrolysed gluten diluted 1:4,000 in 3% (w/v) SMP. The products were coupled 1:20 (product:DIG). Plates were then incubated with 100 µL/well HRP-labelled sheep-anti-DIG-POD (11633716001, Roche Diagnostics GmbH, Mannheim, Germany) diluted 1:1,000 in PBS-T. Plates were washed twice in tap water before development. The values of specific IgE are expressed as log₂ titres with an individual cut-off for each plate according to the positive and negative controls.

Inhibitory ELISA

To examine the competitive capacity for antibody binding of unmodified and acid-hydrolysed gluten, IgG₁ inhibitory ELISA was performed. Maxisorp plates were coated as for indirect ELISA. The test was performed on sera pooled group-wise. Pooled sera were diluted in PBS-T to reach an OD of around 1 in the absence of inhibitor. Sera were pre-incubated for 1 h with a serial 10-fold dilution of the 2 gluten products, individually, as inhibitors. After pre-incubation of sera and gluten products, duplicates of sera/inhibitor mix were added to the plates. Afterwards, this assay followed the same procedure as for the indirect ELISA. The inhibitory ELISA was performed twice. The results are expressed as percentage inhibition against the concentration of the inhibitor.

Avidity ELISA

For measuring the binding strength between the 2 products and the specific IgG₁, avidity ELISA was performed. Maxisorp plates were coated in the same way as for indirect ELISA. Plates were incubated in quadruplicate with 50 µL/well rat serum samples diluted in PBS-T. The dilutions were made to reach an OD of around 1. After incubation with rat sera, plates were incubated for 30 min with 50 µL/well serial 2-fold diluted potassium thiocyanate (KSCN) (P2713, Sigma-Aldrich), starting with a concentration of 4 M. The following procedure was the same as for the indirect ELISA. The results are expressed in percentage inhibition against the concentration of KSCN. The half maximal inhibitory concentration (IC₅₀) was determined for each animal.

Immunoblot

Samples for SDS-PAGE were prepared with 40 µg protein in 2× Laemmli sample buffer (1:1) (1610737, Bio-Rad, CA, USA) and β-mercaptoethanol (1:40) (1610710, Bio-Rad) and heated for 5 min at 95 °C. A mini protean precast gel (Mini PROTEAN TGX stain-free gel, 4568093, Bio-Rad) was loaded with the prepared samples in running buffer (10× Tris/glycine/SDS buffer, 1610732, BIO-RAD). Precision plus unstained standard (1610363, Bio-Rad) was used. The SDS-PAGE was performed at 200 V for 30 min.

Gels were activated in ChemiDoc for 1 min. Subsequently, proteins were transferred to a PVDF membrane using a transfer blot turbo pack (1704156, Bio-Rad). After transfer, the membranes were washed 3 × 5 min in PBS-T and afterwards blocked with 5% (w/v) SMP overnight at 4 °C. Membranes were incubated with pooled sera from the skin sensitisation study diluted 1:100 or 1:1,000 in blocking solution overnight at 4 °C. Membranes were washed 3 × 5 min in PBS-T and incubated for 1 h at RT with secondary antibody, mouse anti-rat IgG₁-HRP diluted 1:1,000 in blocking solution, together with StrepTactin HRP conjugate for visualisation of the standard diluted 1:10,000. Membranes were washed and developed by Clarity Western ECL reagent (1705060, Bio-Rad) for 5 min at RT. Membranes were imaged by ChemiDoc XRS+ (Bio-Rad).

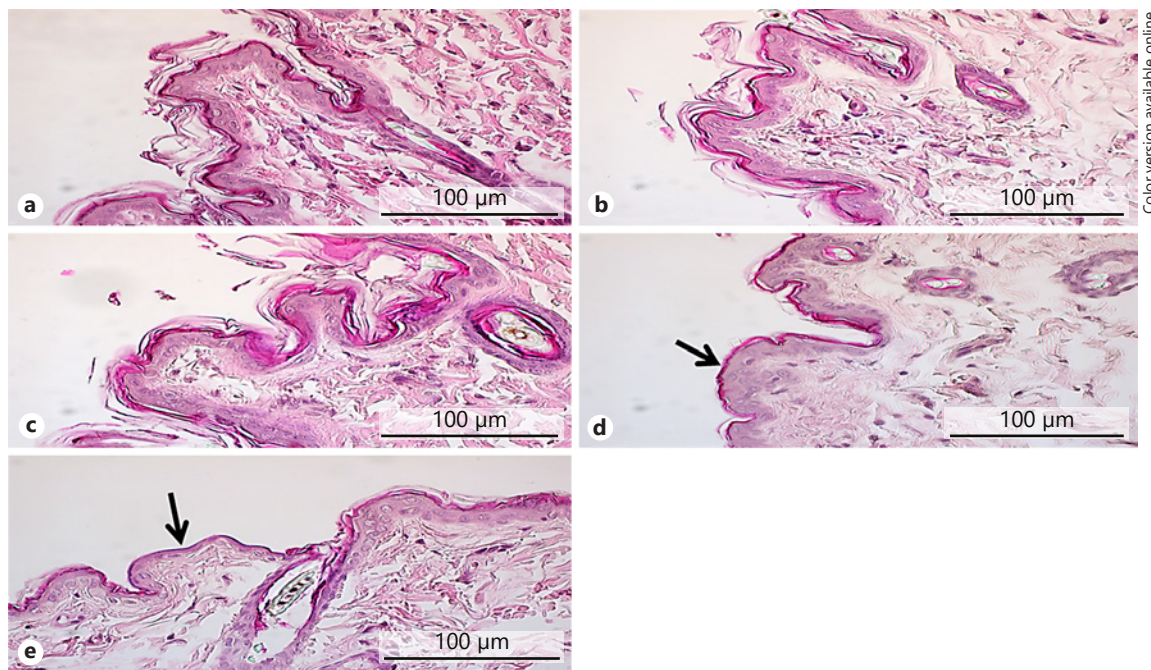


Fig. 2. Skin condition after hair removal. Stained sections of abdominal skin. HE. **a** Untreated. **b** Shaved. **c** Shaved and tape-stripped. **d** Cream-removed hair (arrow shows inner keratin and cell layer). **e** Cream-removed hair and tape-stripped (arrow shows inner keratin layer damaged). Animals were sacrificed immediately after treatment.

Statistical Analysis

Curve analysis and statistical analyses were made using Graph-Pad Prism v7.03 (San Diego, CA, USA). Differences in TEWL were tested for variance with a one-way ANOVA test with multiple comparisons. Curves obtained from avidity and inhibitory ELISA were tested for variance with one-way ANOVA, Bartlett's test for equal variances, and then Tukey's multiple-comparisons test. No statistical significant variances between the curves were obtained, which enabled the calculation of IC_{50} values. The calculated IC_{50} and antibody titre values were examined for group differences using the non-parametric one-way ANOVA Kruskal-Wallis test followed by Dunn's multiple-comparisons test to compare all groups or groups receiving the same product. The Mann-Whitney U test was used for the comparison of 2 groups when testing for differences in duration and dose-dependent response, naïve versus tolerant rats, and avidity. Asterisks indicate statistically significant differences between the given groups: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

Results

Mechanical Disruption of the Skin Barrier Increases the Thickness of the Outer Skin Layer

To develop an animal model where the skin condition could be controlled, pilot studies were conducted to test

the most suitable way of removing hair and slightly damaging the skin. The epidermal layer of the normal rat abdominal skin has a thickness of a few cell layers and no distinct stratum granulosum. The keratin forms a compact thin inner layer (stratum lucidum) and a diffuse outer layer (stratum corneum) (Fig. 2a). Shaving or shaving followed by tape-stripping had little effect on the integrity of the epidermis and on the keratin layer (Fig. 2b, c).

In contrast, removal of hair with depilatory cream, according to the manufacturer's instructions, removed not only the hair, but also the outer keratin layer, leaving the inner keratin and cell layer morphologically intact (Fig. 2d). When skin treated with depilatory cream was tape-stripped, the inner keratin layer was largely removed (Fig. 2e). Immediately after removal of hair with depilatory cream, the skin appeared normal; however, after a couple of hours, the abdominal skin became irritated and the rats started to scratch themselves to a degree that they had to be sacrificed. These results demonstrate that removal of hair with a depilatory cream do not leave the skin barrier intact. In order to be able to control the skin condition and also for ethical reasons, we decided to abandon the use of depilatory cream for the removal of hair.

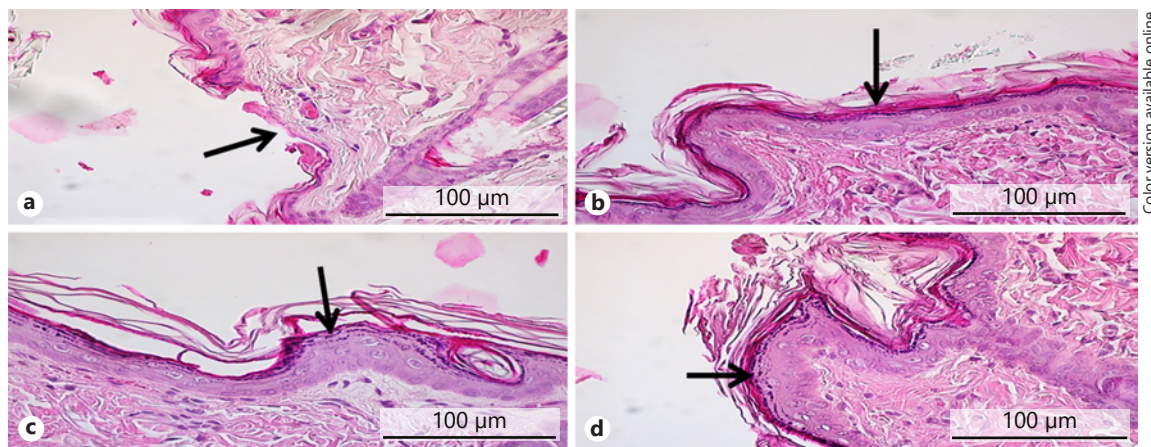


Fig. 3. Skin condition after repeated applications. Stained sections of abdominal skin. HE. **a** Shaved, scratched, and dosed (PBS) rats taken immediately after treatment (arrow shows scratching wound). **b** Shaved, scratched, and dosed (PBS) rats 2 days after the last skin application for 1 week (arrow shows stratum granulosum). **c** Shaved, scratched, and dosed (PBS) rats 2 days after the last skin application for 3 weeks (arrow shows stratum granulosum). **d** Shaved, scratched, and dosed (acid-hydrolysed gluten) rats 2 days after treatment for 5 weeks (arrow shows stratum granulosum).

As we wanted to have a model with a slightly damaged epidermal barrier, a model combining hair removal by shaving followed by scratching with sandpaper was developed. Macroscopically, it was shown that scratching with sandpaper damaged the skin, and, based on histology (Fig. 3a), it was evident that scratching removed the epithelial layer sporadically while leaving most of the epidermis intact, i.e., the desired situation.

The pilot studies included shaving, scratching, and dosing on day 0 followed by dosing on days 1 and 2. This regimen was repeated every week for 5 weeks. Apart from the first 2 animals sacrificed immediately after the first dosing on day 0, all animals were sacrificed 2 days after the dosing period, 2 animals each week. At this point in time, the epidermis was intact, with an inner and outer keratin layer, but also with a very distinct stratum granulosum. This enlargement of the stratum granulosum started already after the first week (Fig. 3b) and continued with similar induction of keratin production in the following weeks (Fig. 3c, d), indicating a thickening of the epidermis as a result of the mechanical stress induced by sandpaper scratching. There was no histological difference between animals dosed with PBS and animals dosed with acid-hydrolysed gluten.

Water evaporation was measured before and after shaving, after scratching (before application), and 1 h after application. In agreement with the histology, no differences could be observed between the 2 groups. Figure 4a shows that, after shaving, no increase in water evapo-

ration was observed whereas after scratching and 1 h after application, the highest increase in TEWL was observed. This pattern was observed on days 0 and 7 with the greatest increase in TEWL seen on day 0. Indeed, according to TEWL, the second bout of scratching did not seem to damage the skin as much as the first did. This correlates very well with the histology where the induction of keratin production could be detected after repeated scratching and application. Looking at individual animals, the majority had no increase in TEWL after shaving whereas a high increase in TEWL was observed after scratching and wrapping (Fig. 4b). Variation between animals could be observed, and in a few animals only a slight increase in water loss was observed after scratching and application (Fig. 4c).

The Rat Skin Sensitisation Model Revealed Duration- and Dose-Dependent Responses

To establish a robust animal model for skin sensitisation, 2 different durations of skin application (i.e., 3 and 5 weeks), 2 different doses (50 and 500 µg), and 2 different post-immunisation regimens (i.p. and oral administration) were studied. The immunogenicity and allergenicity of unmodified and acid-hydrolysed gluten were evaluated by analyses of specific IgG₁ and IgE.

For establishing the animal models for food allergy skin sensitisation, PBS, unmodified gluten, or acid-hydrolysed gluten was applied on the abdominal skin of naïve rats for 3 or 5 weeks (Fig. 1). Analyses of antibody

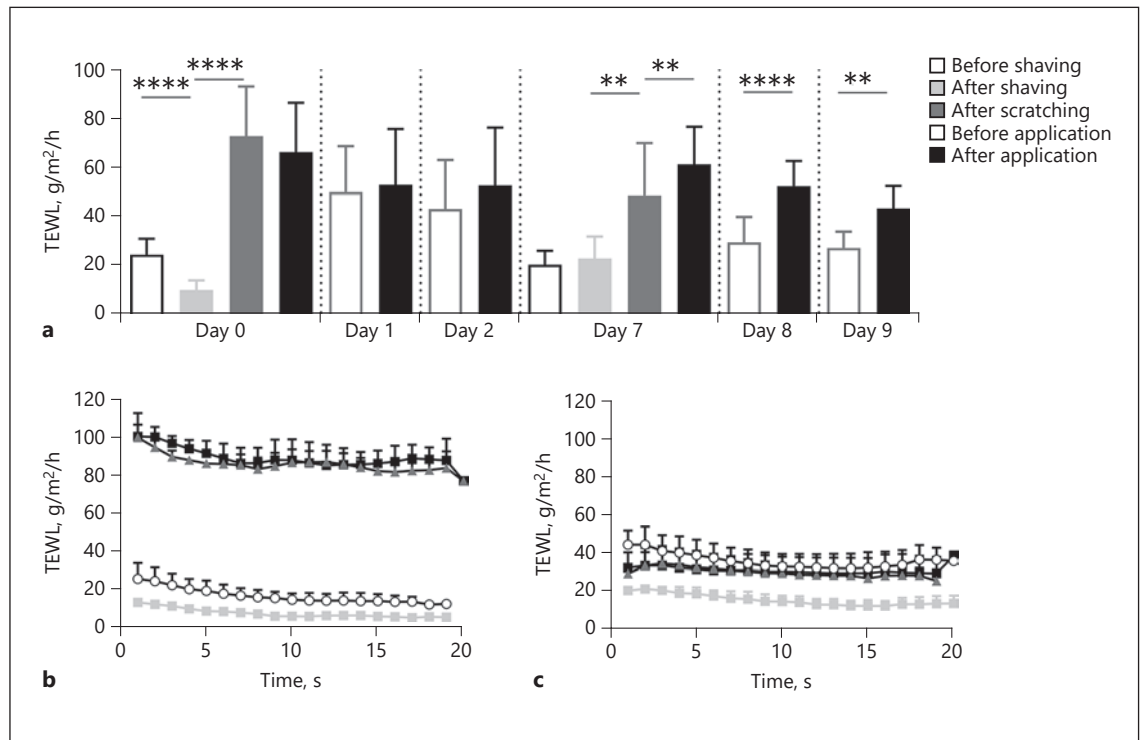


Fig. 4. Water evaporation from the skin measured before and after shaving, after scratching (or before application) and after 1 h of application. **a** Trans-epidermal water loss (TEWL) of 12 animals (both PBS and acid-hydrolysed gluten-dosed animals) from 2 weeks of skin application. **b** TEWL from an individual animal on day 0 representing most animals. **c** TEWL from an individual animal on day 0 with only a slight increase in TEWL representing few animals. Error bars show standard deviation. Statistically significant differences between the indicated groups are shown: ** $p < 0.01$, **** $p < 0.0001$.

responses revealed that both products were able to induce high levels of specific IgG₁, in both the 3- and 5-week dosing periods without post-immunisation (Fig. 5a). Looking at the specific IgE response, both products were able to induce specific IgE antibodies (Fig. 5b, c), although to a higher level after 5 weeks than after 3 weeks of skin application. Results demonstrated that it was possible to sensitise BN rats, without the use of adjuvant, through slightly damaged skin with both the unmodified and acid-hydrolysed gluten product. Different dosing regimens were also studied. Unmodified or acid-hydrolysed gluten was applied on the abdominal skin of the rats at 2 different doses, 50 or 500 µg. The skin application proceeded for 5 weeks, followed by 2 oral gavage. Comparable levels of specific IgG₁ (Fig. 5d) were produced after dosing with 50 and 500 µg. When comparing the doses of 50 and 500 µg, no statistically significant difference was seen with unmodified gluten-specific IgE (Fig. 5e), but there was a statistically significant difference with acid-hydrolysed gluten-specific IgE (Fig. 5f).

Two post-immunisations were given, in order to identify if some animals were primed for sensitisation after skin application without the induction of measurable levels of specific IgE antibodies. Results revealed that, in general, higher levels of specific IgE were seen after 2 post-immunisations compared to skin application alone. Both oral gavage and i.p. post-immunisation were tested, but, surprisingly, a more consistent response was observed after oral gavage which we then decided to use in the following procedures, and the highest concentration (500 µg) and longest duration of application (5 weeks) were chosen in order to compare the sensitising capacity of unmodified and acid-hydrolysed gluten in wheat-naïve versus wheat-tolerant rats.

Naïve Rats Could Be Sensitised to Both Gluten Products whereas Only Acid-Hydrolysed Gluten Was Able to Break the Oral Tolerance of Tolerant Rats

To test the immunogenicity and allergenicity of unmodified and acid-hydrolysed gluten in naïve versus tol-

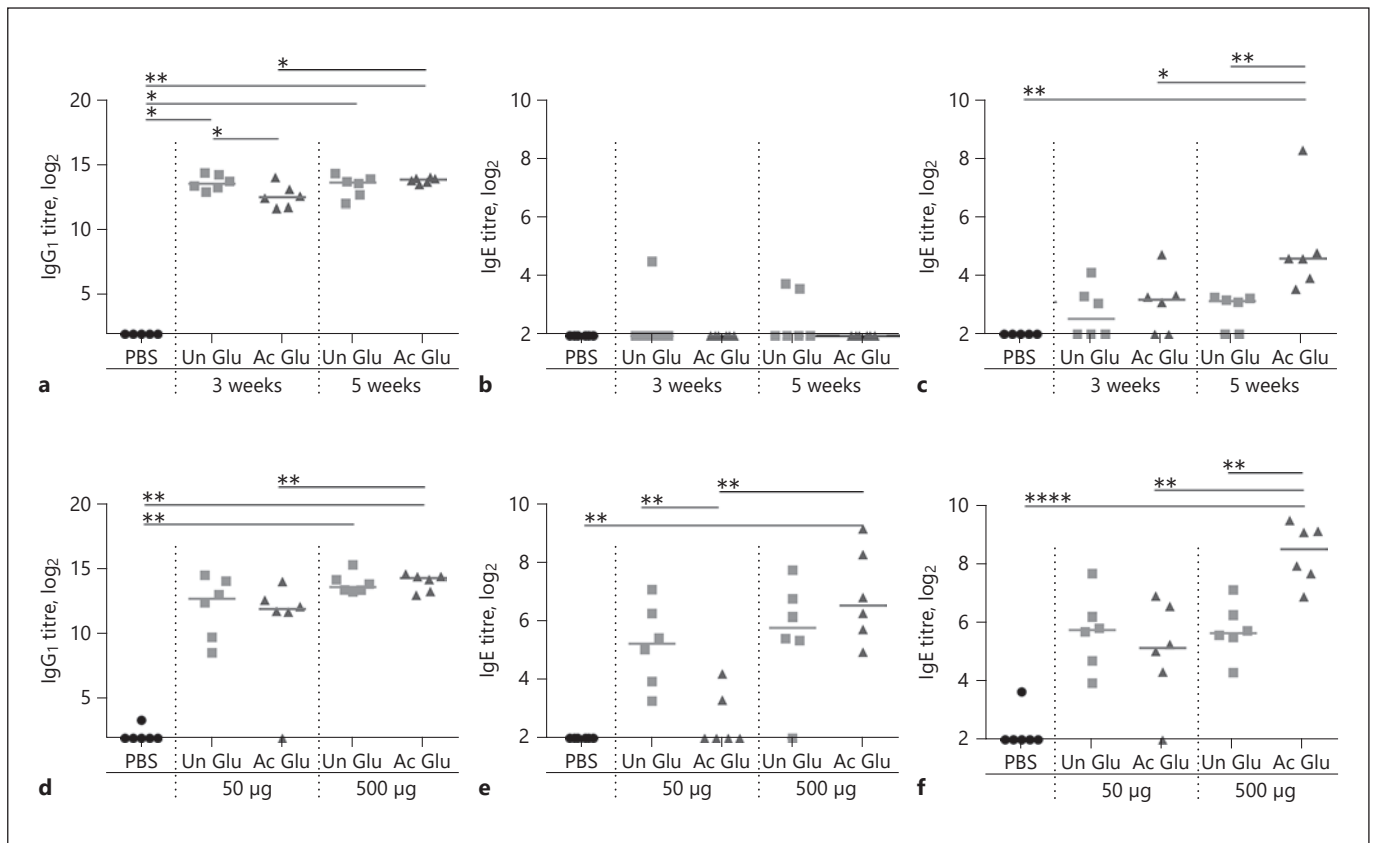


Fig. 5. Comparison of specific IgG₁ and IgE levels. **a–c** Before post-immunisation (day 35). **d–f** After post-immunisation (day 49). **a** Product-specific IgG₁ levels after 3 or 5 weeks of skin application with 500 µg protein. **b** Unmodified gluten-specific IgE after 3 or 5 weeks of skin application with 500 µg protein. **c** Acid-hydrolysed gluten-specific IgE after 3 or 5 weeks of skin application with 500 µg protein. **d** Product-specific IgG₁ after dosing with 50 or 500 µg of gluten product and 2 oral post-immunisations. **e** Unmodified

gluten-specific IgE after dosing with 50 or 500 µg and 2 oral post-immunisations. **f** Acid-hydrolysed gluten-specific IgE after dosing with 50 or 500 µg and 2 oral post-immunisations. Each symbol represents 1 animal and horizontal bars indicate median value. Statistically significant differences between the indicated groups are shown: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Un Glu, unmodified gluten; Ac Glu, acid-hydrolysed gluten.

erant rats after skin application, specific IgG₁ and IgE antibodies were analysed.

In naïve rats, both unmodified and acid-hydrolysed gluten induced a statistically significant specific IgG₁ response after 5 weeks of skin sensitisation (Fig. 6a). In tolerant rats, detectable levels of specific IgG₁ were observed in all groups, irrespective of the application, i.e., PBS, unmodified gluten, or acid-hydrolysed gluten, as a result of wheat-specific antibodies being raised due to the wheat-containing feed. Only the application with acid-hydrolysed gluten induced a slight elevation of specific antibodies, but no statistically significant differences could be observed.

In naïve rats, both the unmodified and the acid-hydrolysed gluten were able to induce specific IgE antibodies

(Fig. 6b, c) and sensitise the rats through the slightly damaged skin without the use of adjuvant. In tolerant rats, only 1 IgE responder was observed, which was in the group dosed with acid-hydrolysed gluten.

The antibody responses after 2 oral post-immunisations revealed a level of specific IgG₁ similar to the level after skin application alone to both naïve and tolerant rats (Fig. 6d). In naïve rats, higher levels of specific IgE (Fig. 6e, f) were evident after 2 post-immunisations for both groups; in tolerant rats, only those dosed with acid-hydrolysed gluten had an increased level of specific IgE. The results clearly reveal that higher levels of specific IgE were induced in naïve rats than in tolerant rats, indicating that naïve rats were easier to sensitise than tolerant rats.

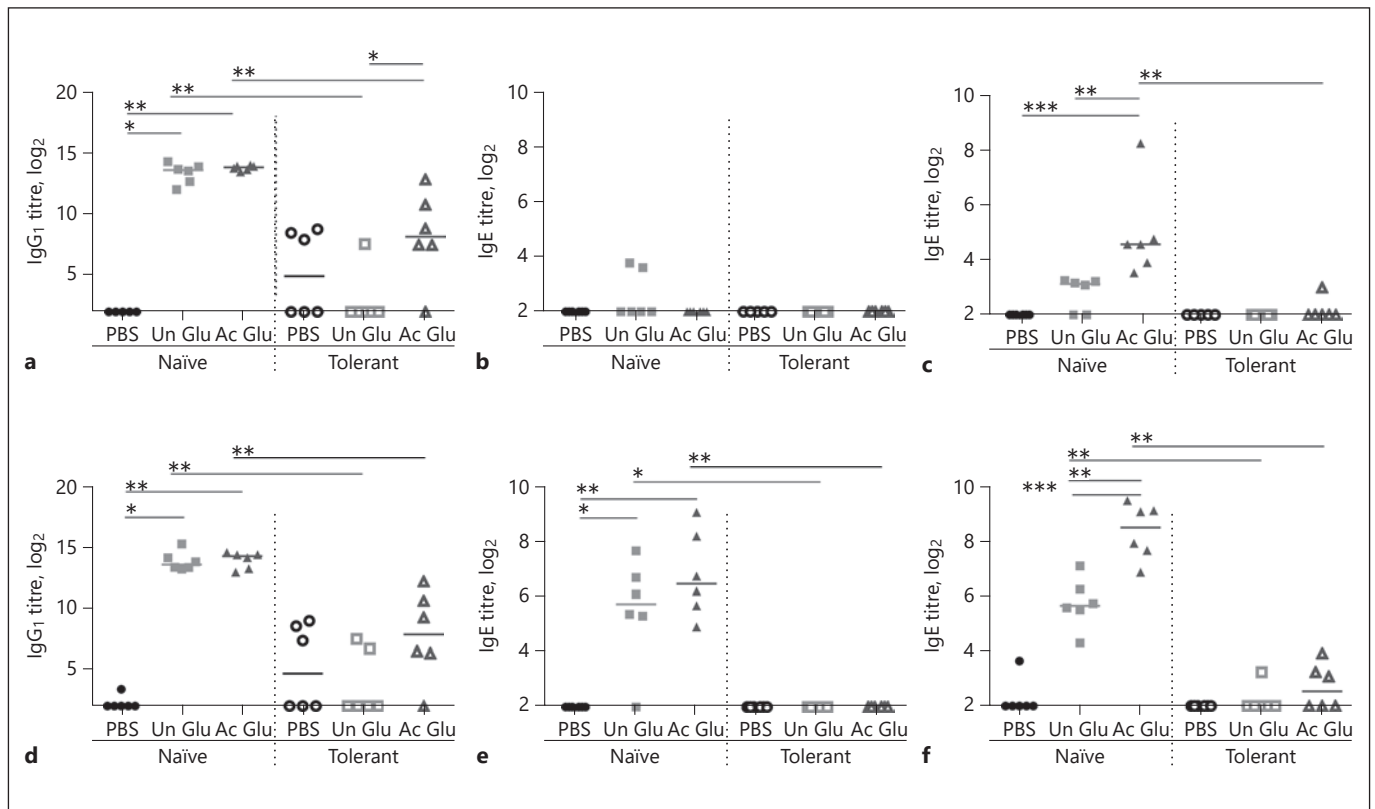


Fig. 6. Comparison of specific IgG₁ and IgE levels in naïve versus tolerant rats. All rats were dosed with 500 µg protein. **a–c** Before post-immunisation (day 35). **d–f** After post-immunisation (day 49). **a** Product-specific IgG₁ after 5 weeks of skin application. **b** Unmodified gluten-specific IgE after 5 weeks of skin application. **c** Acid-hydrolysed gluten-specific IgE after 5 weeks of skin application. **d** Product-specific IgG₁ after 2 oral post-immunisations.

e Unmodified gluten-specific IgE after 2 oral post-immunisations. **f** Acid-hydrolysed gluten-specific IgE after 2 oral post-immunisations. Each symbol represents 1 animal and the horizontal bar indicates a median value. Statistically significant differences between the indicated groups are shown: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Un Glu, unmodified gluten; Ac Glu, acid-hydrolysed gluten.

To identify whether there might be differences in the binding pattern of antibodies raised in tolerant versus naïve rats, and between the antibodies raised against unmodified and acid-hydrolysed gluten, immunoblotting was performed. This revealed differences in the binding pattern between tolerant and naïve rats, irrespective of whether the rats were dosed with unmodified (Fig. 7b, d) or acid-hydrolysed gluten (Fig. 7c, e). For example, in the tolerant rats dosed with acid-hydrolysed gluten, two bands were more pronounced, around 75 and 30 kDa, than in the naïve rats. In the naïve rats, one larger region was more pronounced, in the area around 50 kDa, than in the tolerant rats. This indicates that epitope binding varies and protein reactivity differs in tolerant and naïve rats. Differences in binding patterns were also seen in rats dosed with unmodified gluten and those dosed with acid-hydrolysed gluten, irrespective of whether the products

were applied to the tolerant or naïve rats. This indicates that antibodies were developed against different epitopes and that the protein reactivity profiles differed between the 2 gluten products.

In order to analyse the cross-reactivity between the 2 gluten products, inhibition ELISA was performed. Figure 8a shows that acid-hydrolysed gluten was capable of fully inhibiting the binding between unmodified gluten and the IgG₁ antibodies raised against the unmodified gluten. This could only be performed with serum from naïve rats due to the low number of antibodies in tolerant rats. In contrast, unmodified gluten was not able to fully inhibit the binding between the acid-hydrolysed gluten and IgG₁ antibodies raised against acid-hydrolysed gluten (Fig. 8b). This was most pronounced for IgG₁ antibodies raised in tolerant rats, where unmodified gluten was only capable of reaching a maximum inhibition of

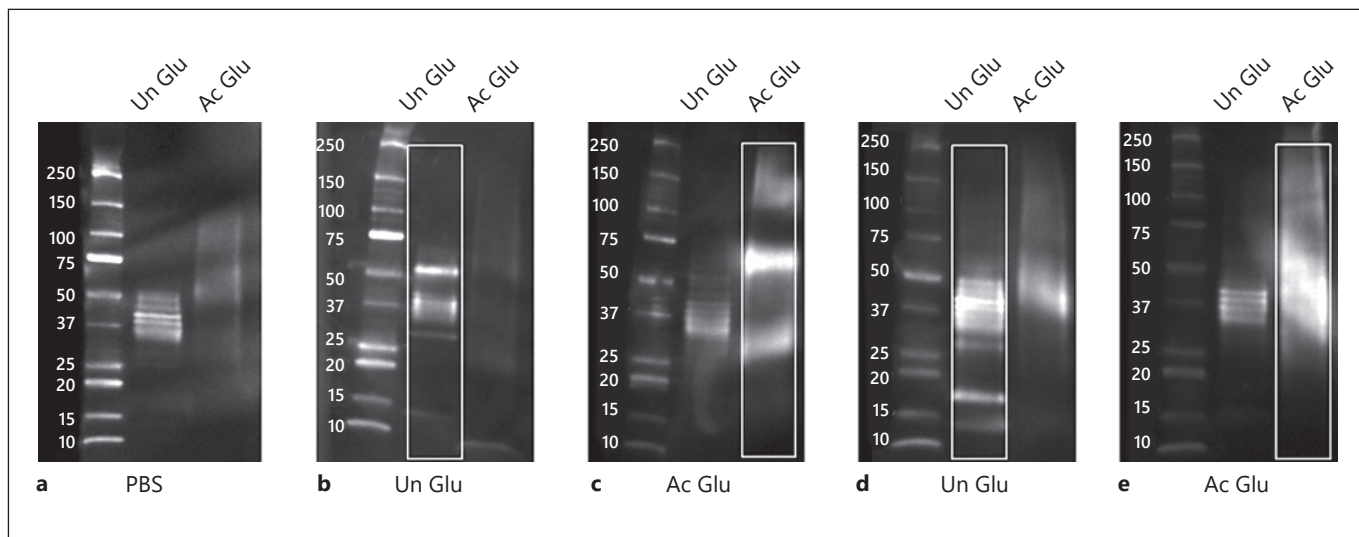


Fig. 7. IgG₁ immunoblot. Comparison of IgG₁-binding pattern to unmodified gluten and acid-hydrolysed gluten. SDS-PAGE was performed with the 2 gluten products, and proteins were transferred to a PVDF membrane. Membranes were incubated with serum pools from tolerant (**a–c**) and naïve (**d, e**) rats dosed on the skin with PBS (**a**), 500 µg unmodified gluten (**b, d**) or 500 µg acid-hydrolysed gluten (**c, e**), followed by 2 oral post-immunisations. Un Glu, unmodified gluten; Ac Glu, acid-hydrolysed gluten.

approximately 35%. This clearly demonstrates differences between the 2 products, indicating that, while all epitopes on gluten were maintained in acid-hydrolysed gluten, new epitopes were additionally developed after acid hydrolysis. Differences between naïve and tolerant rats indicated that more antibodies are developed against epitopes not present on unmodified gluten in tolerant rats than in naïve rats.

The binding strength of the induced antibodies was evaluated by a KSCN avidity ELISA which showed that the binding strength was slightly higher after 2 oral post-immunisations (on day 49) than after skin application alone (on day 35) (Fig. 8c). Additionally, it can be seen that the antibody-binding strength of acid-hydrolysed gluten was lower than that of unmodified gluten, as the avidity measures revealed that a lower level of KSCN was needed for the half inhibition of binding to acid-hydrolysed gluten when compared to unmodified gluten, irrespective of which antibodies were raised against unmodified or acid-hydrolysed gluten (Fig. 8c, d). When testing the antibodies raised against the unmodified gluten and the acid-hydrolysed gluten towards the same gluten products, similar avidity was observed (Fig. 8d), indicating that antibodies raised against unmodified gluten and acid-hydrolysed gluten have a similar binding capacity.

Discussion

BN rats are well-recognised as high IgE responders that, to some extent, resemble atopic humans in their predisposition to develop allergy, which enables the investigation of allergic responses and the mechanisms behind them in this specific animal strain [32]. In this study, it was shown that BN rats provided a suitable animal model for studying food allergy skin sensitisation. Unmodified and acid-hydrolysed gluten were able to sensitise naïve BN rats after application on slightly damaged skin, but only acid-hydrolysed gluten could induce a slightly higher IgE response in wheat-tolerant BN rats, and to a much lower degree.

In the pilot studies, the skin condition was examined in relation to hair removal and product application. Using Veet as depilatory cream seemed effective, but undesired alterations occurred. There appear to be heterogeneous conclusions about using Veet as a depilatory cream. Results presented by Dunkin et al. [33] indicated no obvious changes of the skin barrier when using Veet. Similar conclusions were presented by Tordesillas et al. [34], who confirmed that skin was intact after Veet application. Examination of the skin condition is relevant as the degree of skin damage may heavily influence sensitisation.

In this study, mechanical disruption of the skin was performed to resemble individuals with impaired skin

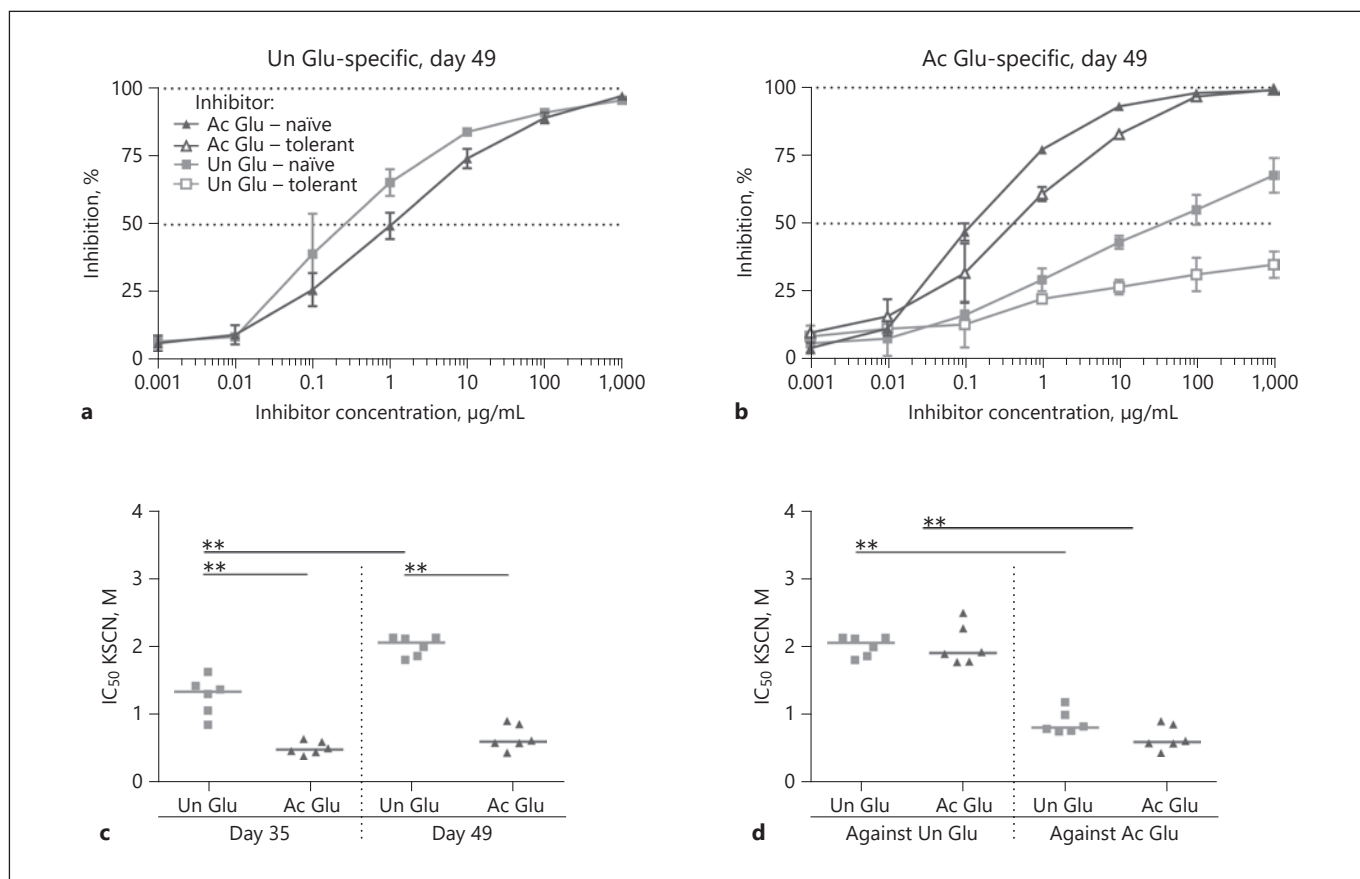


Fig. 8. IgG₁ antibody-binding capacity of the 2 gluten products. **a** Serum pools from naïve rats dosed with 500 μg unmodified gluten, and then post-immunised, were pre-incubated with 10-fold dilutions of unmodified or acid-hydrolysed gluten. IgG₁ antibody levels were too low for tolerant rats dosed with unmodified gluten to perform inhibitory ELISA. **b** Serum pools from naïve and tolerant rats dosed with 500 μg acid-hydrolysed gluten, and then post-immunised, were pre-incubated with 10-fold dilutions of unmodified or acid-hydrolysed gluten. Results are expressed as the percentage inhibition against the concentration of the inhibitor. **c** Comparison of IC₅₀ values from avidity measurements from in-

dividual rats after 5 weeks of skin application (day 35) and after 2 post-immunisations (day 49). Avidity was tested against the same product that the rats were dosed with. **d** Comparison of IC₅₀ values from avidity measurements from individual rats after 2 post-immunisations. Avidity was tested against unmodified gluten and acid-hydrolysed gluten. Each symbol represents 1 animal and the horizontal bar indicates a median value. Statistically significant differences between the indicated groups are shown: * $p < 0.05$, ** $p < 0.01$. Un Glu, unmodified gluten; Ac Glu, acid-hydrolysed gluten.

barrier functions. A change in the skin barrier may have an influence on sensitisation through the skin, as it may allow the entry of antigens otherwise excluded by an intact skin barrier. Mouse models of food allergy skin sensitisation commonly use tape-stripping to cause mechanical disruption of the skin. In this study, no influence of tape-stripping on shaved skin was observed, which entailed scratching with sandpaper. Alteration of the skin barrier in atopic dermatitis is evident from the reduced water content in the stratum corneum and by increased TEWL [35]. In our pilot study, we observed a clear correlation between skin histology and water evaporation

when examining the skin after scratching and repeated exposure to the products.

Applying products on the skin with occlusive patches may influence the permeability of the skin and thereby enhance the allergen penetration. In several mouse studies, occlusive patches have been used for allergen application. Patches have been left for 24 h [26, 36] or a longer period of exposure such as 3 days [37, 38]. In this study, the gluten products were left on the skin for 1 h, a short period of allergen exposure similar to the 40 min used in the study by Wavrin et al. [39]. These short periods of allergen exposure may correspond more closely to what

consumers experience and may not alter the permeability of the skin. However, it is difficult to conclude any strict correlation between the duration of allergen exposure and the sensitisation response.

In this study, a robust animal model was established for studying the sensitising capacity of 2 different gluten products through the skin. Acid-hydrolysed gluten has been shown to cause reactions in humans. Here, we show the differences between the 2 products, unmodified and acid-hydrolysed gluten, transferred to the BN rat model.

By including duration- and dose-dependent sensitisation, various degrees of immune responses could be observed, revealing a higher risk of sensitisation, the higher the dose applied on the skin and the longer the duration of the dosing regimen for sensitisation.

The sensitising capacity of unmodified and acid-hydrolysed gluten was evaluated both in naïve and tolerant BN rats. Naïve rats resemble individuals with a naïve immune system without prior contact to the gluten or cross-reactive products, while tolerant rats resemble individuals with an established oral tolerance to gluten due to prior oral exposure. In naïve rats, both products were able to induce allergic sensitisation through the skin whereas in tolerant rats, only acid-hydrolysed gluten was able to induce specific IgE. It has previously been shown in a mouse study by Strid et al. [24] that applying peanut protein onto the skin was able to partly break existing oral tolerance against peanut. This might be in correlation with the cases from Japan where allergic skin reactions caused by acid-hydrolysed wheat protein have contributed to breaking the oral tolerance in individuals previously tolerant to wheat [22, 40]. These observations correspond very well with our results.

Evaluating the differences in immune response towards the 2 gluten products, the antibody-binding capacity was examined by immunoblotting and inhibitory ELISA of pooled serum from rats sensitised with either unmodified or acid-hydrolysed gluten. The results clearly indicate that acid hydrolysis of gluten induces new epitopes while still maintaining the original ones. These findings are in line with the results presented by Kroghsbo et al. [30] who found that rats sensitised i.p. or orally with acid-hydrolysed gluten developed specific IgG₁ responses with a binding capacity different from that in rats sensitised with unmodified gluten. This is in agreement with Denery-Papini et al. [41], who found that IgE from patients allergic to deamidated gluten had the strongest reaction, with an epitope where 2 or 3 of 4 glutamines were changed to glutamate due to deamidation.

Differences in the development of antibodies against new epitopes can also appear in naïve and tolerant rats. Different binding patterns were observed from the immunoblotting and a lower inhibition capacity of unmodified gluten was seen in tolerant rats dosed with acid-hydrolysed gluten. Additionally, the avidity ELISA results indicated that the newly formed epitopes bound antibodies with a lower avidity than the shared epitopes did. In conclusion, both gluten products were able to sensitise through slightly damaged skin in naïve BN rats. However, the results indicate that only the acid-hydrolysed gluten was able to break an already established oral tolerance after skin exposure.

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Statement of Ethics

Ethical approval for animal experiments was given by the Danish Animal Experiments Inspectorate and the authorisation number given 2015-15-0201-00553-C1. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.R.B. participated in study design, laboratory analyses, data analysis and manuscript writing. C.B.M. participated in study design, data analysis, manuscript writing, and critical revision of the manuscript. K.L.B. participated in study design, data analysis, manuscript writing and critical revision of the manuscript. All authors read and approved the final manuscript.

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