The use of aluminum hydroxide as adjuvant modulates the specific antibody response - a Brown Norway rat study with native and denatured cow's milk allergens

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Running title: Al(OH)₃ modulates antibody specificity

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Abbreviations: 3D, Three-dimensional; ALA, α -lactalbumin; Al(OH)₃, Aluminum hydroxide; BLG, β -lactoglobulin; BN, Brown Norway; CMA, Cow's milk allergy; DIG, Digoxigenin; I.p., Intraperitoneally; IT, Immunotherapy; OD, Optical density; RT, Room temperature.

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Abstract

There is a need for efficient methods to treat food allergy, however, no immunotherapeutic method has yet been satisfactory due to the high rate of unpredictable severe reactions and the limited efficacy. Therefore, modified versions of food allergens have been suggested as alternatives to the parent proteins for immunotherapy.

The aim of the study was to compare the inherent allergenicity of the native and denatured version of the cow's milk proteins β -lactoglobulin and α -lactalbumin, and to study the impact of the use of $\text{Al}(\text{OH})_3$ as an adjuvant.

Brown Norway rats were immunised intraperitoneally with either native or denatured β -lactoglobulin or α -lactalbumin, with or without the use of $\text{Al}(\text{OH})_3$ as adjuvant. Antibody responses were analysed in various ways by means of different ELISAs.

Both the immunogenicity and the sensitising capacity of the cow's milk allergens were influenced by their globular folding, with the native version being more allergenic than the denatured counterpart. The native folded proteins mainly raised antibodies against conformational epitope, whereas the denatured versions predominantly raised antibodies against linear epitopes. Most interestingly, the study showed that the use of $\text{Al}(\text{OH})_3$, besides increasing immunogenicity and sensitising capacity of the cow's milk allergens, caused a modification of the specificity of the antibodies raised against the native version of the proteins. Adsorption of the native forms of the allergens to $\text{Al}(\text{OH})_3$ caused a significant greater proportion of antibodies raised against linear epitopes, stressing that the adsorption induced a partly unfolding of the proteins. This may have implications for IT safety and efficacy.

Keywords: Milk allergy, Epitopes, Adjuvant

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Conflict of Interest

The authors declare no conflict of interest

Introduction

Food allergy is defined as ‘an adverse health effect arising from a specific immune response that occurs reproducible on exposure to a given food’.¹ Allergies to food have recently been estimated to affect up to 5% of adults and 8% of children,^{1,2} and appears to be a major health problem of growing concern.^{1,3} Cow’s milk allergy (CMA) affects around 2.5% of infants and young children, and is the most common form of food allergy in children.⁴⁻⁶

Living with food allergy has a huge impact on the quality of life of children.^{7,8} At the moment no cure exists for food allergy and avoidance of the offending food is generally the only reliable management option.^{9,10} However, for cow’s milk allergic infants an alternative approach to strict avoidance is the use of hypoallergenic infant formulas.¹¹ In addition, incorporation of heat-denatured milk into the diet has recently been suggested as an alternative to strict avoidance,^{12,13} as studies have shown that milk-containing baked products can be tolerated by the majority of milk allergic children.¹²⁻¹⁴ In children who were able to add milk-containing baked products into their diet, immunologic changes similar to the ones induced by oral immunotherapy (IT) were observed¹⁴ and suggested to speed up recovery from CMA.¹⁵⁻¹⁷ Therefore, treatment with heat-denatured milk products has been suggested as a new IT approach.^{13,18} Due to the high risk of unpredictable severe adverse reactions upon food allergy IT with native allergens, alternative approaches are highly desirable.

The three-dimensional (3D) protein structure defines the amino acid residues on the surface of an allergen available for antibody binding. Thus, the accessibility of a given epitope may greatly be influenced by the structural folding of the allergen. Antibody-binding epitopes are divided into linear and conformational epitopes, based on the proximity of the amino acids in the primary structure of the protein that are involved in the antibody binding. Epitopes formed by a continuous stretch of amino acids are defined as linear epitopes, while epitopes formed by amino acids brought together by the secondary, tertiary or quaternary folding of the protein but distant from each other in the primary sequence are defined as conformational epitopes.^{19,20} Proteins in their native form, generally, possess both conformational as well as linear epitopes, whereas denatured proteins only possess linear epitopes, due to the unfolding and thereby loss of their native structure. Denaturation of proteins may therefore lead to a reduced allergenicity compared to its native counterpart, due to the destruction of the conformational epitopes, though new linear epitopes may be exposed which were previously buried inside the globular protein. This is what happens during extensive heating of milk, such as during baking.^{18,21} Such hypoallergenic allergen

derivatives with reduced allergenicity, could allow for the use of larger doses in IT compared to its native counterpart, and at the same time will allow for a more safe approach with reduced risk of inducing severe allergic side effects.^{13,22,23}

Traditionally, adjuvant is a common constituent of IT vaccines, where aluminum hydroxide ($\text{Al}(\text{OH})_3$) is standard use.²⁴⁻²⁶ Although the mechanisms underlying the adjuvanticity of $\text{Al}(\text{OH})_3$ is far from being fully understood, adsorbance of allergens to $\text{Al}(\text{OH})_3$ is widely used due to the repository effect and to the increase in immune response towards the co-formulated allergen. $\text{Al}(\text{OH})_3$ is associated with the induction of a Th2 response, thought to be a result of direct stimulation of antigen presenting cells, complement cascades and chemokines, leading to higher and more persistent production of specific antibodies against the co-formulated allergen.^{24,25} Further, the use of $\text{Al}(\text{OH})_3$ is a mainstay approach in animal sensitisation models.^{27,28}

Animal models are unique tools allowing for the validation of safety and efficacy of new IT approaches in a controlled environment, before entering human trials. Especially, when modification of allergens are introduced and changes in the structure of the allergen are the consequences, animal models allowing for studying the potential of *de novo* sensitisation due the possibility of newly introduced epitopes, are of high value.²⁹ Therefore, in the present study, we investigated the inherent allergenicity of two cow's milk proteins, β -lactoglobulin (BLG) and α -lactalbumin (ALA), in their native as well as denatured form, in a Brown Norway (BN) rat model of food allergy. To study the impact of the use of $\text{Al}(\text{OH})_3$ as an adjuvant, BN rats were immunised intraperitoneally (i.p.), with the native and denatured version of BLG and ALA, respectively, with or without adsorbance to this adjuvant.

Materials and Methods

Allergens

BLG was from a pilot batch purified and kindly provided by Arla Foods Ingredients (Videbæk, Denmark) and ALA (61289, Fluka, Sigma, St. Louis, MO, USA) was from Sigma.

Denaturation of allergens

Samples of the cow's milk allergens BLG and ALA as well as samples of digoxigenin (DIG)-coupled BLG and ALA were concentrated by evaporation in SpeedVac. A solution of 6 M guanidine-HCl (G4505, Sigma), 0.5 M Tris-HCl (A1087.1000, AppliChem, Darmstadt, Germany) and 0.01 M EDTA (1.08418, Merck, Darmstadt, Germany) pH 8.6 was added to the allergens to obtain an allergen concentration of 5 mg/mL. Subsequently dithiothreitol (DTT, D0632, Sigma) was added to give a final concentration of 0.1 M and the solutions were saturated with nitrogen, capped and incubated for 2 h at 50 °C. Iodoacetamide (I1149, Sigma) diluted in 0.5 M Tris-HCl to a concentration of 0.6 M, pH 8.6 was added to the solution to give a final concentration of 0.24 M. After incubation for 30 min at room temperature (RT) 2-mercaptoethanol (M7522, Sigma) was added to give a final concentration of 2.4 M. Lastly, the solutions were placed in dialysis-tubes with a pore size of 6-8 kDa (Spectra/Por®Dialysis Membrane MWCO: 6-8000, Spectrum Laboratories, Inc., Rancho Domingues, CA, USA), and dialysed against PBS (137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.2) at 4 °C and afterwards stored at -20 °C until further use.

Native PAGE

To test the degree of modification of BLG and ALA, a native gel electrophoresis was performed using a Criterion TGX Tris-HCl Leammli-like 10-20% gel (567-1113, Bio-Rad, Hercules, CA, USA). Native PAGE was performed with the native and denatured samples of the two allergens in pure and DIG-coupled form (~2-5 µg), essentially as described in Madsen et al.³⁰

Animals

At an age of four weeks, BN rats from the in-house breeding colony (National Food Institute, Technical University of Denmark, Denmark) were weaned. They were housed in macrolon cages (3 per cage), with a 12 hour light:dark cycle, at a temperature of 22 ± 1 °C and a relative humidity

of $55 \pm 5\%$. Rats were observed twice daily and clinical signs were recorded. Rats were kept on a diet free of milk for at least 10 generations to avoid tolerance to the studied allergens. Diet and acidified water (pH 3.5) were given *ad libitum*. Animal experiments were carried out at the National Food Institute facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate and the authorisation number given 2009/561-1710. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

I.p. study with native and denatured BLG and ALA with or without the use of adjuvant

To study the sensitising capacity of ALA and BLG from cow's milk in both their native and denatured form and to examine the influence of $\text{Al}(\text{OH})_3$ as an adjuvant on the antibody specificity, BN rats 4-8 weeks of age were allocated into eight groups of six rats ($n = \text{three per sex}$). The rats were immunised i.p. three-times on days 0, 14 and 28 with 200 μg of: 1) native BLG, 2) denatured BLG, 3) native ALA or 4) denatured ALA without the use of adjuvant or with 200 μg of 5) native BLG, 6) denatured BLG, 7) native ALA or 8) denatured ALA with the use of $\text{Al}(\text{OH})_3$ (Alhydrogel 2%, Brenntag Biosector A/S, Frederikssund, Denmark) as adjuvant. The choice of this immunisation regimen was based on a previous study³⁰, and on the aim of raising an antibody response without use of adjuvant, while still being able to compare the different allergens. The immunisation solutions were prepared by combining 0.25 mL of PBS containing 200 μg of allergen, with 0.25 mL of Alhydrogel 2%, resulting in 12.5 mg $\text{Al}(\text{OH})_3/1 \text{ mg}$ of allergen), and incubated overnight at 4 °C, in order to allow the allergens to adsorb to the $\text{Al}(\text{OH})_3$. One week after the last immunisation rats were sacrificed and blood was collected. See Fig. 1 for an overview of the animal experimental design.

ELISA for detection of BLG and ALA specific IgG1

In order to test the specific IgG1 response to native as well as denatured BLG, and ALA, ELISAs were performed, as previously described.³⁰

Antibody-capture ELISA for detection of BLG and ALA specific IgE

In order to test the specific IgE response to native as well as denatured BLG and ALA, antibody-capture ELISAs were performed, as previously described.³⁰

Inhibitory ELISA

Inhibitory ELISAs were performed in order to examine the competitive capacity of native and denatured cow's milk allergens for binding to IgG1 raised in the BN rats. ELISA procedure was as described for detection of specific IgG1, with the exception of sera being preincubated with the inhibitor antigen solution. Serum from individual rats were diluted to given an optical density (OD) of approximately 1.0 and incubated with 10-fold serial dilutions of native BLG, denatured BLG, native ALA or denatured ALA (0.0005-500 µg/mL). Serum-inhibitor mixtures as well as serum without inhibitor antigen (control) were added to plates in duplicates, coated with the same antigen solution as the give rats were immunised with. Results are expressed as percentage of inhibition for a given concentration of inhibitor.

Statistical analysis

ELISA results expressed as Log₂ antibody titres were examined for group differences, using the non-parametric one-way ANOVA, Kruskal-Wallis test, followed by Dunn's multiple comparison of three or more groups. For comparison of two groups the non-parametric Mann-Whitney test was used. Differences between groups of animals were regarded as significant when $P \leq 0.05$. Asterisks indicate a statistically significant difference between two given groups: $= P \leq 0.05$, $** = P \leq 0.01$, and $*** = P \leq 0.001$.

Results

In the present study, antibodies raised against purified BLG or ALA in their native or denatured form, with or without the use of $\text{Al}(\text{OH})_3$ as adjuvant were analysed for their reactivity against the native as well as denatured form (of the respective allergens) by means of different ELISAs. In the following sections, results are combined in various ways in order to address different issues.

Immunogenic and sensitising capacity of native versus denatured allergens

In order to compare the inherent immunogenicity as well as sensitising capacity of the native and denatured form of the two cow's milk allergens, specific IgG1 and IgE responses were measured against the same form of the allergen as they were raised against in rats dosed without use of adjuvant. Results showed that the native as well as denatured form of the two cow's milk allergens contained immunogenicity as well as sensitising capacity. Results showed that there were no statistically significant differences between the inherent immunogenicity and sensitising capacity of the native and the denatured BLG, though the sensitising capacity seemed reduced when the protein was denatured (Fig 2 A and C). In contrast, for ALA, the denatured form showed a statistically significant reduced immunogenicity compared to its native counterpart. This seems also to be the tendency for the sensitising capacity, where only 2 out of 6 rats were sensitised to the denatured ALA compared to all 6 rats for the native ALA. However, those two rats sensitised to denatured ALA, reacted with production of high IgE levels (Fig 2 B and D).

Impact of the use of $\text{Al}(\text{OH})_3$ on immunogenicity and sensitising capacity of the allergens

To study the effect of the use of adjuvant on the antibody levels, specific IgG1 and IgE responses were measured against the same variant of the allergen as they were raised against in rats immunised with or without adjuvant. In general the use of $\text{Al}(\text{OH})_3$ as adjuvant increased the immunogenicity as well as sensitising capacity of both allergen, irrespective of whether they were presented to the immune system in their native or denatured form, though not all conditions showed statistically significant difference between rats dosed with or without the use of adjuvant (Fig 3). Not only did the adjuvant raise the antibody levels, it also did decrease the heterogeneity of responses within groups of rats.

Linear versus conformational epitopes of native versus denatured allergens

Denaturation of proteins will result in the disruption of the native 3D structure and ideally in the unfolding of the protein. Thus, while linear epitopes are maintained, there will be a consequential loss of conformational epitopes. Therefore, analysing the antibody reactivity against the native as well as the denatured versions of the allergens will allow for examination of the relative ratio between antibodies raised against linear and conformational epitopes. For antibodies raised against the native allergens, a lower antibody binding capacity was shown when measured against the denatured compared to native version of the allergens (Fig 4). This demonstrates an inherent capacity of both BLG and ALA to induce antibodies primarily against conformational epitopes. However, the relative proportion of antibodies reacting with denatured (linear epitopes) compared to native (linear and conformational epitopes) allergen differed between the two allergens. From the differences in mean serum dilution factors ($2^{\text{titre value}}$) giving a specific response towards native versus denatured allergens for each individual groups of rats, the relative proportion of linear versus conformational epitopes was calculated. IgG1 raised against native BLG, showed an approximate ratio of 1:15 for antibodies raised against linear versus conformational epitopes (Fig 4A), while IgE showed an approximate ration of 1:50 (Fig 4C). For ALA an even higher proportion of antibodies were raised against conformational epitopes, with a ratio of linear versus conformational epitopes for IgG1 of approximately 1:480 (Fig 4B). IgE raised against native ALA, were only able to bind native ALA, as no response was evident against denatured ALA, stressing the native ALA-specific IgE epitopes were only of the conformational version (Fig 4D). In contrast to the native version of the two cow's milk allergens, which primarily induced antibodies against conformational epitopes, the denatured version of the proteins only raised antibodies directed against linear epitopes, as no reduction in the antibody response against the denatured compared to the native form of the allergens was shown (Fig 4). In fact, for IgG1 as well as IgE raised against the denatured form of the allergen, for both BLG and ALA, the antibody binding capacity was greater for the denatured compared to the native version of the allergens. This indicates that antibodies raised against the denatured allergens were not only directed against linear epitopes accessible on the native form of the allergens, but also against so-called neo-epitopes, parts of the allergens not accessible in the native form of the proteins. Results indicated that such neo-epitopes were of greater importance for ALA compared to BLG and greater for IgE compared to IgG1, though differences were small.

Impact of the use of $Al(OH)_3$ on the antibody specificity

In order to study the impact of the use of adjuvant on the antibody specificity, the proportion of linear versus conformational epitopes was compared between antibodies raised with or without the adjuvant $\text{Al}(\text{OH})_3$. For antibodies raised against native allergens, a great difference in the ratio of linear versus conformational epitopes was seen between rats dosed with and without adjuvant. For BLG the ratio was reduced from 1:15 to 1:3 for IgG1 (Fig 4A) and from 1:50 to 1:15 for IgE (Fig 4C). Such reduction was even more pronounced for ALA where the use of $\text{Al}(\text{OH})_3$ led to a reduction in the ratio from 1:480 to 1:5 for IgG1 (Fig 4B). For IgE raised against native ALA, the use of adjuvant resulted, in the ability of antibodies to react with the denatured ALA (Fig 4D). So, while IgE raised against native ALA without the use of $\text{Al}(\text{OH})_3$ only reacted with conformational epitopes of ALA, the use of $\text{Al}(\text{OH})_3$ led to the recognition of linear epitopes as well. Such impact on the antibody specificity by the use of adjuvant was not seen for antibodies raised against the denatured allergens. For antibodies raised against denatured allergens, the relative proportion of antibodies reacting with the native versus the denatured version of the allergens was more or less identical except for IgG1 raised against denatured ALA. For antibodies raised against denatured BLG, the increase in reactivity to denatured BLG compared to native BLG was exactly the same with and without the use of adjuvant for both IgG1 and IgE (Fig 4A and C). For IgG1 raised against denatured ALA, the use of adjuvant increased the importance of neo-epitopes two-fold (Fig 4B), while for IgE the use of adjuvant did not change the relative proportion of antibodies reacting with native compared to denatured ALA, but increased the amount of rats developing antibodies reacting with only the denatured ALA (Fig 4D).

Competitive capacity of native versus denatured allergens for antibody binding and the influence of the use of adjuvant

In order to compare the competitive capacities of native and denatured allergens for IgG1 binding and to study the influence of whether rats were dosed with native or denatured allergen and whether rats were dosed with or without the use of adjuvant, inhibitory ELISAs were performed. For rats dosed with native BLG (Fig 5A), native BLG clearly showed a greater competition capacity compared to denatured BLG, irrespective of whether antibodies were raised against native BLG adsorbed to $\text{Al}(\text{OH})_3$ or not. This indicates a loss of epitopes upon denaturation of BLG. However, the use of adjuvant greatly influenced the competitive capacities of native as well as the denatured BLG for IgG1 binding. A reduction in the competitive capacity for IgG1 binding was seen for the native BLG when antibodies were raised with the use of adjuvant in comparison

to antibodies raised without adjuvant. In contrast, an increase in competitive capacity for IgG1 binding was seen for the denatured BLG when rats were dosed with the use of adjuvant when compared to antibodies raised without adjuvant. This confirms the results from the ELISAs, showing a greater importance of antibodies directed against linear epitopes when rats were dosed with native BLG adsorbed to $\text{Al}(\text{OH})_3$.

For antibodies raised against denatured BLG (Fig 5B), differences in competitive skills for native and denatured BLG were not that obvious, though denatured BLG showed a bit stronger competitive capability than native BLG, indicating that some neo-epitopes had emerged due to the denaturation process. This correlated well with results obtained by the ELISAs. While denatured BLG had similar competitive skills, whether antibodies were raised with or without $\text{Al}(\text{OH})_3$, native BLG showed lower competitive skills when antibodies were raised with adjuvant, indicating that adsorption of denatured BLG to $\text{Al}(\text{OH})_3$ changed the structure of denatured BLG even further away from native BLG than did the denaturation alone.

Similarly to BLG, when antibodies were raised against native ALA (Fig 5C), irrespective of the native ALA was adsorbed to $\text{Al}(\text{OH})_3$ or not, the competitive skills for binding to the IgG1 were much greater for native than for denatured ALA. This difference was much more pronounced than for BLG, correlating well with result from the ELISAs, showing a much greater impact of denaturation for ALA than for BLG. When antibodies were raised with the use of adjuvant, a reduction in the competitive capacity for native ALA was seen, though it was not as pronounced as for BLG. In contrast, denatured ALA were only able to compete for antibody binding when antibodies raised against native ALA were raised with the use of $\text{Al}(\text{OH})_3$. This indicated that the use of $\text{Al}(\text{OH})_3$ somehow changed the structure of native ALA to a structure more similar to denatured ALA, revealing epitopes identical to those exposed in denatured ALA.

For antibodies raised against denatured ALA (Fig 5D), difference in competitive skills of native and denatured ALA were much less than when antibodies were raised against native ALA, irrespective of antibodies were raised with or without adjuvant, a pattern similar to BLG. However, in contrast to denatured BLG, competitive capabilities of native ALA were similar for antibodies raised with or without adjuvant. On the other hand, a great difference in competitive skills were seen for denatured ALA, dependent on whether antibodies were raised with or without adjuvant, where denatured ALA showed a much greater competitive capacity for antibodies raised against denatured ALA with the use of $\text{Al}(\text{OH})_3$.

Discussion

Search for new hypoallergenic variants of food allergens is in focus, due to the high risk of severe adverse side effects during food allergy IT with native allergens. Denaturation of proteins, whether it is by heat-treatment or chemically, is an approach that potentially reduces the allergenicity.^{12-14,30-33} In the present study we investigated the allergenicity of two denatured cow's milk proteins in comparison to their native form. Only a small decrease in the inherent immunogenicity and sensitising capacity was evident for denatured BLG in comparison to the native form. In contrast, a significant reduction in the inherent immunogenicity and sensitising capacity was evident for denatured ALA compared to its native counterpart. A reduced allergenicity is in line with previous studies investigating the sensitising capacity of denatured variants of food allergens, showing that the sensitising capacity of the Brazil nut allergen Ber e 1 and that the sensitising capacity of the peach allergen Pru p 3 were reduced after denaturation.^{31,33} Collectively, these studies also indicate that the degree of reduction in immunogenicity as well as sensitising capacity for denatured versions of allergens, in comparison to their native counterparts, differs between allergens. This could indicate that for some proteins the primary structure contains some sensitising capacity, which is the case of BLG, whereas for other proteins the sensitising capacity is more a result of the secondary and tertiary folding, which seems to be the case for ALA.

For antibodies raised against the native version of the allergens, the denatured variant showed a significantly reduced antibody binding capacity compared to the native form. While the binding capacity was greatly reduced for IgG1, the IgE binding capacity was almost completely abrogated. Both for the IgG1 and the IgE binding capacity, the reduction was most pronounced for ALA; results which were confirmed by inhibitory ELISA. This confirms our previous study, likewise showing a more pronounced reduction in the antibody binding capacity after denaturation for ALA in comparison to BLG, when rats were i.p. immunised with native proteins.³⁰ Other studies investigating cross-reactivity between native and denatured version of allergens showed that for β -casein only a marginal reduction in antibody binding capacity was evident,³⁰ whereas a significant reduction in antibody binding capacity was seen for the denatured peanut Ara h 2³² and an almost complete abrogation of the antibody binding capacity was seen for the denatured peach allergen Pru p 3.³³ This indicates that the reduction in antibody binding capacity for denatured versions of allergens varies greatly between different proteins, and that such difference may be related to differences in the structural folding of the native protein, where globular proteins show a higher degree of decrease in allergenicity than flexible unstructured proteins. This is directly translated to

the similarity or difference in structure of proteins in their native versus denatured form, which again can be translated into the ratio of linear versus conformational epitopes of native proteins. This study showed that both native BLG and ALA by far induced most antibodies directed against conformational epitopes, though ALA showed the greatest importance of conformational epitopes. Whether denatured food allergens could be promising candidates for food allergy IT, with a higher safety and preserved efficacy profile than the parent protein, is yet to be demonstrated. Nevertheless, results from animal models suggest that the heat-treated versions of foods could retain the ability to induce tolerance and yet have a better safety profile when used for OIT due to denaturation of the proteins.^{34,35} However, while clinical studies have shown that heat-treated milk is well tolerated by most CMA patients, it may not be a more safe and efficacious strategy in milk allergic patients sensitive to heat-treated milk.^{13,36}

This point to the fact that in contrast to allergies induced through the skin or respiratory tract, the situation is a bit more complicated for allergies induced via the gastrointestinal tract, where the allergens are modified during the digestion process, as proteins are subjected to structural changes and degradation due to the acidic and proteolytic environment.³⁷ This influences the protein structure recognised by the immune system, and the relative proportion of antibodies raised against linear versus conformational epitopes.

Al(OH)₃ is the most commonly used chemical adjuvant and is a constitute of many IT vaccines.²⁵ Further, Al(OH)₃ is the most applied adjuvant in i.p. sensitisation studies in animal models of food allergy.^{27,28} In both situations, Al(OH)₃ is used to increase the immune response towards the co-formulated antigen. In concordance with this, the present study showed that both the immunogenicity as well as the sensitising capacity of the proteins were increased with the use of Al(OH)₃, irrespective of the specific allergen and its globular folding or lack of such. However, of greater importance is that the use of Al(OH)₃ seemed to change the structural folding of the protein and consequently presenting a modified version of the protein to the immune system, resulting in changed specificity of the antibodies raised against the protein. It has previously been shown that protein adsorption to Al(OH)₃ may induce conformational changes in the protein structure, dependent on the given protein and the amount of protein adsorbed,^{38,39} though it is also stated that adsorption of proteins to Al(OH)₃ maintain and stabilise the protein, as part of the repository effect.^{25,40} As clearly shown in the present study, the adsorption of native allergens to Al(OH)₃ induced a significant reduction in the ratio of antibodies raised against linear versus

conformational epitopes, which was not the case for the denatured version of the allergens, stressing that adsorption of allergens to $\text{Al}(\text{OH})_3$ induces a partly unfolding of the proteins. Whereas the use of $\text{Al}(\text{OH})_3$ reduced the ratio from 1:15 to 1:3 for BLG specific IgG1, the use of $\text{Al}(\text{OH})_3$ reduced the ratio from 1:480 to 1:5 for ALA specific IgG1. This implies that the proteins co-formulated with the $\text{Al}(\text{OH})_3$ in vaccines or in animal studies are presented to the immune system in a somewhat modified version and may therefore not induce antibodies with the same specificity as the protein would without being co-formulated with $\text{Al}(\text{OH})_3$. As shown in this study, this is a concern only with structurally folded proteins, as the denatured proteins do not show the same pattern. Further, it is seen that the effect is somewhat larger for the more compact folded ALA compared to BLG. It has previously been shown that proteins co-formulated with $\text{Al}(\text{OH})_3$ induced a modified specificity in the raised antibody response, but not that this was a result of partly denaturation of the proteins.⁴¹ The structural integrity of proteins used in IT vaccines is of great importance, as the resulting immune response and thereby specificity of induced antibodies has a huge impact of the therapeutic efficacy. Therefore, co-formulation of proteins with $\text{Al}(\text{OH})_3$ may induce sub-optimal IT vaccines for prophylactic approaches.

In allergy IT where the treatment is mostly undertaken with the offending food or a derivative hereof in already allergic individuals, adsorption of proteins to $\text{Al}(\text{OH})_3$ and a consequential loss of structure, may have implication for both the safety and the efficacy profile, though this has not been investigated.⁴² IT with proteins adsorbed to $\text{Al}(\text{OH})_3$ may to a certain extent mimic the use of heat-treated or chemically induced denatured proteins. In an animal study, investigating the allergenicity of the grass pollen proteins with or without the use of $\text{Al}(\text{OH})_3$ as adjuvant, it was seen that co-formulation reduced the histamine release. This could suggest that $\text{Al}(\text{OH})_3$ -induced modification of proteins may provide a more safe version of the allergen for IT.

In conclusion, the present study showed that adsorption of allergens to $\text{Al}(\text{OH})_3$ caused a modulation of the antibody responses against the allergen. This may have implications for IT safety and efficacy and for the interpretation of animal immunisation experiments using $\text{Al}(\text{OH})_3$.

Author Contributions

KLB and CBM designed and executed the animal experiment. MSA performed all lab analyses.

KLB drafted the whole manuscript. KLB, MSA and CBM reviewed the manuscript.

Data Availability

Data available on request from the authors

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Figure legends

Figure 1. Animal experimental design. BN rats (6 per group, 3 per sex) were immunised i.p. three-times on days 0, 14 and 28 with 200 µg of: 1) native BLG, 2) denatured BLG, 3) native ALA or 4) denatured ALA without the use of adjuvant or with 200 µg of 5) native BLG, 6) denatured BLG, 7) native ALA or 8) denatured ALA with the use of Al(OH)₃ as adjuvant. Rats were sacrificed at day 35 and blood was collected for analyses. Pictures are from Colourbox

Figure 2. Immunogenicity and sensitising capacity. Inherent immunogenicity of native versus denatured BLG (A) or ALA (B), and the inherent sensitising capacity of native versus denatured BLG (C) or ALA (D). The specific IgG1 and IgE responses were measured against the same antigen as the rats were immunised with. Each symbol represents an individual rat immunised three times with the indicated antigen, and horizontal lines indicate the median value in each group of rats. Statistically significant differences between indicated groups are shown with asterisks. $**P < 0.01$.

Figure 3. Comparison of immune responses of cow's milk proteins with and without use of adjuvant. Immunogenicity of native versus denatured BLG (A) or ALA (B) with or without Al(OH)₃, and sensitising capacity of native versus denatured BLG (C) or ALA (D) with or without Al(OH)₃. Specific IgG1 and IgE responses were measured against the same antigen as the rats were immunised with. Each symbol represents an individual rat immunised three times with the indicated antigen, and horizontal lines indicate the median value in each group of rats. Statistically significant differences between indicated groups are shown with asterisks. $**P < 0.01$.

Figure 4. Comparison of antibody responses against native and denatured cow's milk proteins. Sera from BN rats immunised i.p. with native or denatured BLG or ALA with or without adjuvant were assessed for binding against the native or denatured version of the antigens in order to evaluate the relative importance of linear versus conformational epitopes to which the antibodies were raised. IgG1 from rats dosed with native or denatured BLG (A) or ALA (B) with or without adjuvant was measured against native versus denatures BLG and ALA, respectively. IgE from rats dosed with native or denatured BLG (C) or ALA (D) with or without adjuvant was measured against native versus denatures BLG and ALA, respectively. Approximate ratios of

antibodies reacting with native (closed symbols) versus denatured (open symbols) antigens are indicated as relative ratio of linear versus conformational epitopes for each group of rats. Each symbol represents an individual rat, and horizontal lines indicate the median value in each group of rats.

Figure 5. Comparison of competitive capacities of native versus denatured cow's milk proteins. The inhibitory capacity of native versus denatured BLG, for binding to IgG1 raised in rats immunised three times with native BLG (A) or denatured BLG (B) with or without the use of adjuvant. The inhibitory capacity of native versus denatured ALA, for binding to IgG1 raised in rats immunised three times with native ALA (C) or denatured ALA (D) with or without the use of adjuvant. Serum samples from individual rats were preincubated with 10-fold dilutions of the native or denatured version of the allergen it was immunised with. Results are presented as the percentage inhibitory capacity, when competing for IgG1 binding with the form of the allergen as the rats were immunised with. Error bars in the inhibition curves represent SD when pooling results from all rats within each group.

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