



Influence of protein expression system on elicitation of IgE antibody responses: Experience with lactoferrin

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ABSTRACT

With increased interest in genetically modified (GM) crop plants there is an important need to understand the properties that contribute to the ability of such novel proteins to provoke immune and/or allergic responses. One characteristic that may be relevant is glycosylation, particularly as novel expression systems (e.g. bacterial to plant) will impact on the protein glycoprofile. The allergenicity (IgE inducing) and immunogenicity (IgG inducing) properties of wild type native human lactoferrin (NLF) from human milk (hm) and neutrophil granules (n) and a recombinant molecule produced in rice (RLF) have been assessed. These forms of lactoferrin have identical amino acid sequences, but different glycosylation patterns: hmNLF and nNLF have complex glycoprofiles including Lewis (Le)^x structures, with particularly high levels of Le^x expressed by nNLF, whereas RLF is simpler and rich in mannose residues. Antibody responses induced in BALB/c strain mice by intraperitoneal exposure to the different forms of lactoferrin were characterised. Immunisation with both forms of NLF stimulated substantial IgG and IgE antibody responses. In contrast, the recombinant molecule was considerably less immunogenic and failed to stimulate detectable IgE, irrespective of endotoxin and iron content. The glycans did not contribute to epitope formation, with equivalent IgE and IgG binding recorded for high titre anti-NLF antisera regardless of whether the immunising NLF or the recombinant molecule were used substrates in the analyses. These data demonstrate that differential glycosylation profiles can have a profound impact on protein allergenicity and immunogenicity, with mannose and Le^x exhibiting opposing effects. These results have clear relevance for characterising the allergenic hazards of novel proteins in GM crops.

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1. Introduction

There remains continuing need for the safety assessment of novel gene products engineered as novel traits into crop plants (Fischer et al., 2012; Goodman and Tetteh, 2011). In this context a major concern is that the novel protein may have inherent allergenic potential (Bolt and Hengstler, 2010; Lack et al., 2002). Allergenicity is a legitimate and important safety issue, given the prevalence of food allergy (Wang and Sampson, 2011), and the serious potential health consequences.

It must be acknowledged that food proteins vary substantially in their immunogenic and allergenic potential and that a normal diet results in exposure to many thousands of proteins. Almost all of these are potentially immunogenic, and a small proportion has the ability, in susceptible subjects, to cause IgE-mediated allergic sensitisation (Platts-Mills, 2012). The need is therefore to distinguish between allergenic and non-allergenic proteins and for this purpose a stepwise approach is currently favoured. This

includes consideration of the source of the transgene, identification of sequence homology or structural motifs common to known allergenic proteins, and the measurement of the resistance of proteins to proteolytic digestion (Goodman and Tetteh, 2011; Mondal et al., 2011).

The continuing refinement of that paradigm and the identification of new and improved approaches will be founded on an understanding of the characteristics of proteins that confer on them the potential to cause allergic sensitisation. There are several properties that have been associated with allergenicity including: aggregation, enzymatic activity and stability to heat and proteolysis (Breiteneder and Mills, 2005; Huby et al., 2000). One important characteristic that has received comparatively little attention in this context is glycosylation. Many common food allergens are glycosylated, including the peanut allergen *ara h 1*, the brazil nut allergen *ber e 1*, the chicken egg allergens *gal d 1* and *2* (ovomucoid and ovalbumin) and the kiwi fruit allergen *act d 4* (Allergome, 2012; Kaulfürst-Soboll et al., 2011a). It is also known that certain sugar residues can act as IgG and IgE binding sites for both IgG and IgE antibodies on plant proteins (Altmann, 2007). Of particular relevance with respect to safety assessment is that the same transgene protein expressed in different host systems will likely

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display varying patterns of glycosylation, and if such differences impact on the allergenicity of the protein then this may complicate hazard characterisation. Thus, for example initial studies are frequently conducted with recombinant proteins derived from bacterial expression systems that lack the potential for glycosylation. Consequently the immunological properties of such proteins may not be reflective of the same gene product expressed subsequently in the host plant. In fact it has also been claimed that altered glycosylation patterns associated with different host plant species can impact on the immunogenicity and allergenicity of a protein (Prescott et al., 2005).

In the current investigations we have made use of the availability of different forms of the iron-binding protein, lactoferrin (LF). Due to its anti-microbial and bacteriostatic properties human LF has been manufactured for various applications using several expression vectors, including bacterial, fungal, yeast, mammalian and plant systems (Gonzalez-Chavez et al., 2009). As an endogenous protein, human LF has no history of allergenicity in man, whereas bovine LF (in cows' milk) is an important food allergen (Gaudin et al., 2008) that displays considerable (~80%) homology with other mammalian LF (Nozaki et al., 2003). However, the purpose of the experiments described herein was to examine the impact of differential glycosylation per se on the development of immune and allergic responses rather than to correlate the reactivity of individual proteins in mice with their ability to cause allergy in humans. Thus, the availability of differentially glycosylated forms of human LF was the primary reason for selection of this protein rather than the direct relevance of this particular protein for allergy in humans. It has been shown that native LF from isolated from human breast milk and from neutrophils exhibits a complex glycoprofile, including sialic acid, fucose, mannose and Lewis (Le) x and y/b (Le^x and Le^{y/b}) structures (Graham et al., 2011), whereas the rice recombinant species is rich in mannose and contains the plant specific glycans xylose and arabinose (Fujiyama et al., 2004). We have therefore compared immune (IgG and IgE antibody production) responses provoked by the native molecule isolated from both human breast milk and from neutrophils with the recombinant material produced in rice. Resistance to proteolytic digestion has been examined also.

2. Materials and methods

2.1. Mice

Female (8–12 wk old) BALB/c strain mice obtained from Harlan Seralab (Loughborough, Leicestershire, UK) were used throughout these investigations. Mice were housed on sterilised wood bedding with materials provided for environmental enrichment. Food (Beekay Rat and Mouse Diet No1 pellets; B&K Universal, Hull, UK) and water were available *ad libitum*. The ambient temperature was maintained at $21 \pm 2^\circ\text{C}$ and relative humidity was $55 \pm 10\%$ with a 12 h light/dark cycle. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 under a Home Office approved project licence.

2.2. Test materials

Human milk native LF (hmNLF; apo and Fe-saturated [holo]; Sigma, St Louis, MO, USA), human neutrophil native LF (nNLF; Sigma), recombinant human LF expressed in rice (*Oryza sativa*; RLF; Seracare Life Sciences Milford, MA, USA) and β lactoglobulin from cows' milk (β LG; Sigma) were used in these studies. All proteins were >99% pure with respect to protein and 90% pure with respect to total protein versus salt content. Samples were analysed for endotoxin content by quantitative limulus amebocyte assay according to the manufacturer's instructions (Lonza, Wokingham, UK).

2.3. Pepsin digestion assay

The method used was based upon the multi-laboratory study published by Thomas et al. (2004). In brief, each test protein (0.1 mg/ml) was incubated with simulated gastric fluid (SGF) (0.084 N HCl, 35 mM NaCl, pH 1.2, and 4000 units of porcine pepsin [Sigma]) at 37°C . Aliquots were removed after 0–60 min of incubation, immediately quenched with $5\times$ Laemmli buffer (40% glycerol, 5% β -mercaptoethanol [BME], 10% sodium dodecyl sulphate [SDS], 0.33 M Tris, 0.05% bromophenol blue,

pH 6.8) and 200 mM NaHCO₃ pH 11 and placed at $>75^\circ\text{C}$ for 10 min. Controls for pepsin auto-digestion (pepsin without test protein) and test protein stability controls (reaction buffer with test protein but without pepsin) were included. Aliquots of these control samples were taken at 0 and 60 min only. Samples were stored at -20°C prior to analysis by SDS-PAGE.

2.4. SDS-PAGE electrophoresis

Samples were subjected to SDS-PAGE electrophoresis under reducing conditions, using 10% polyacrylamide peptide gels with a tricine buffer (100 mM Tris, 100 mM Tricine, 0.1% SDS pH8.2; Biorad Laboratories, Hercules, CA, USA) system. Mark 10 standard markers (Biorad Laboratories) containing 10 proteins ranging in size from 10 to 250 kDa were included on all gels. Gels were fixed for 5 min in 5% trichloroacetic acid, followed by washing in 45.5% methanol/9% glacial acetic acid for 1 h, stained in Coomassie biosafe brilliant blue solution (Biorad Laboratories) for 10 min and destained in water. Gels were scanned using a Li-cor gel scanner (Li-cor Biotechnology, Lincoln, NE, USA) and densitometric analysis carried out using Odyssey[®] software (Li-cor). Integrated intensity (intensity and area of band following subtraction of background) and the percentage change in band density compared with the time zero value were calculated.

2.5. Sensitisation for antibody responses

Groups of mice ($n=5-10$) received 0.25 ml of various % (w/v) of the different LF proteins in phosphate buffered saline (PBS) by intraperitoneal injection on days 0 and 7. Ten mice per group were required to provide sufficient serum for analyses using both homologous and heterologous substrates. In some experiments, animals received 0.25 ml of 1% RLF in the presence or absence of *Escherichia coli* (E. coli) 055:B55 lipopolysaccharide (LPS) (Sigma; 1.2×10^6 endotoxin units [EU]/mg) by intraperitoneal injection on days 0 and 7. Fourteen or 28 days after the initiation of exposure, all mice were exsanguinated by cardiac puncture. Individual and pooled serum samples were prepared and stored at -70°C until analysis.

2.6. Measurement of anti-protein IgG and IgG1 antibody responses

Protein-specific IgG and IgG1 antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as described previously (Dearman et al., 2003). Plastic microtitre plates (Nunc, Copenhagen, Denmark) were coated with 100 $\mu\text{g}/\text{ml}$ of protein in PBS overnight at 4°C . The plates were blocked with 2% bovine serum albumin (BSA) in PBS at 37°C for 30 min. Serial doubling dilutions of pooled or individual mouse serum samples in 1% BSA in PBS were added and plates incubated for 3 h at 4°C . Plates were incubated for 2 h at 4°C with horse radish peroxidase labelled sheep anti-mouse IgG (1 in 4000) or rat anti-mouse IgG1 (1 in 1000) antibody (antibodies from Serotec, Kidlington, Oxfordshire, UK). Enzyme substrate (*o*-phenylenediamine) was added and the reaction stopped by the addition of 0.5 M citric acid. Substrate conversion at OD450 nm was measured using an automated reader. Titre was determined as the maximum dilution of serum at which an OD450 nm reading of >0.5 was achieved. Negative control (naïve) serum readings never exceeded this reading. Data are displayed as OD450 nm titration curves or following logarithmic transformation as mean and SE log₂ antibody titres.

2.7. Measurement of anti protein IgE antibody responses

The presence of protein specific IgE antibodies was detected by passive cutaneous anaphylaxis (PCA) assay as described previously (Dearman et al., 2003). For IgE responder frequency, individual serum samples were analysed; IgE titres were determined using pooled serum samples. Individual serum samples (neat) or pooled serum samples were injected (30 μl) into the dermis of the ears of naïve recipient mice ($n=2$ or 4, respectively). Two days later, 0.25 mg of lactoferrin and 1.25 mg of Evans blue dye (Sigma) in 0.25 ml PBS were injected intravenously. Thirty min later, mice were terminated and the diameter of the cutaneous reaction measured. A positive (IgE) response was recorded if the challenge resulted in a >3 mm blue lesion in the skin in the majority of recipient animals (pooled serum samples), with antibody titre recorded as the highest dilution of serum resulting in a positive PCA reaction. For individual serum samples, the serum sample was identified IgE positive if the mean diameter of the challenge-induced cutaneous reaction was >3 mm.

2.8. Statistical analyses

Comparisons between responses to the different forms of LF with respect to IgG1 and IgE antibody titres were analysed following logarithmic transformation (log₂). For direct comparisons between forms of LF for IgE titre, Students' *t* test was used (no detectable IgE was assigned the value of zero post transformation). For multiple comparisons (more than two groups), one-way ANOVA followed by Bonferroni post hoc test was utilised. Comparisons between the numbers of IgE responders and non-responders were analysed using contingency tables (Fishers exact). All statistical tests were carried out using GraphPad Prism ($*p < 0.01$, $**p < 0.001$; GraphPad, San Diego, CA, USA).

3. Results

3.1. Differential antibody responses are induced by native milk and recombinant rice LF

The immunogenicity and allergenicity of hmNLF and rice LF were investigated. In three independent experiments mice were immunised with hmNLF or RLF (1%) and the production of antigen specific IgG1 and biologically active IgE antibody was measured. Mice exposed to hmNLF produced vigorous anti-LF IgG1 (Fig. 1) antibody responses, whereas the recombinant protein was considerably less immunogenic, provoking significantly lower titres ($p < 0.01$). In initial analyses, antibody responses were measured by ELISA using homologous substrates; anti-sera raised against hmNLF was analysed using hmNLF substrate and anti-sera provoked by administration of RLF was assessed using RLF substrate. In order to investigate the contribution of the sugar residues to the IgG antibody binding sites, repeat analyses were conducted using the heterologous substrates in the ELISA (Fig. 1). These investigations revealed that the IgG1 binding activity was independent of substrate utilised in the ELISA, thus identical high titres were achieved for anti-hmNLF anti-sera whether analyses were conducted using the homologous or heterologous protein as substrate. Similarly, relatively low titres were recorded for anti-RLF anti-sera regardless of which form of LF was used in the ELISA analysis. Identical patterns were recorded for anti-LF IgG antibody responses (data not shown). The IgE content of the sera was determined by PCA for each of the three independent experiments, again using the homologous substrates. In each experiment, NLF stimulated marked IgE responses (titre 1 in 32), whereas the recombinant material failed to induce detectable IgE (titre < 1) in any of the 3 independent experiments. Identical results were achieved when heterologous protein was used in the challenge phase of the PCA assay (high titre anti-hmNLF IgE and no detectable anti-RLF IgE antibody), demonstrating that the carbohydrate groups were not contributing to the IgE antibody binding sites. Furthermore, the lack of IgE antibody provoked by the recombinant material was not simply a function of the kinetics of induction of antibody. If serum was drawn 28 days, rather than the standard 14 days, after the initiation of exposure, vigorous IgE responses were recorded still following administration of hmNLF (titre of 1 in 32), whereas there was no detectable IgE antibody recorded following treatment with the recombinant form (titre < 1). IgG1 and IgE antibodies can be distinguished on the basis of their differential heat stability (IgE is labile at 56 °C) and affinity for mast

cell receptors, with IgG1 antibodies dissociating more quickly than do IgE antibodies (active after 2 h and 48 h, respectively) and this forms the basis of the PCA assay (Lehrer and Vaughan, 1976). For selected anti-NLF antisera, heat treatment (3 h at 56 °C) was shown to abrogate PCA responses (data not shown).

3.2. Differential immunogenicity and allergenicity of NLF and rice RLF is not due to differential iron or endotoxin content

Many functions of LF have been attributed to its ability to bind iron (Baker and Baker, 2009) so the role of iron binding status in the induction of antibody responses was investigated. In subsequent experiments responses to RLF (0.1% Fe) and the apo form of hmNLF (0.1% Fe) used in the preliminary experiments were compared with those provoked by an iron saturated (holo) form of hmNLF that was 0.6% iron bound (Fig. 2). BALB/c strain mice received intraperitoneal injections of the proteins (1%) and antigen specific IgG1 and IgE antibody was measured using in each case the immunising protein in the analyses. Titration curves for IgG1 antibody responses (Fig. 2A) revealed that antibody activity was independent of iron saturation status. Thus, the titration curves generated for pooled anti-sera isolated after exposure to the apo and holo forms of hmNLF were very similar whereas much lower responses were recorded for the RLF. Similarly, analyses of IgG1 antibody titres for individual serum samples revealed that both forms of hmNLF induced vigorous and relatively high titre IgG1 antibody whereas RLF stimulated significantly weaker responses (Fig. 2B). The antibody binding profiles were independent of the form of LF used in the analyses (data not shown). Furthermore, the iron status of hmNLF did not impact on its ability to stimulate marked IgE antibody responses (Fig. 2C)

Another property of proteins that may impact on antibody responses is the extent of endotoxin contamination (Eisenbarth et al., 2002). All preparations of LF used in these experiments had similar low levels of endogenous endotoxin (< 10 EU/mg). In order to rule out low level endotoxin as a factor, and also to determine the adjuvant effects of exogenous endotoxin, mice were exposed to 1% RLF alone or in the presence of exogenous LPS at 1 µg/ml or 100 µg/ml (equivalent to 1.2×10^3 EU or 1.2×10^5 EU, respectively). Control mice received 1% hmNLF alone. As recorded previously, hmNLF alone resulted in more vigorous IgG1 antibody responses than did RLF alone, with a clear differential between the two antibody titration curves observed for serum samples pooled on a treatment group basis (Fig. 3A). Concurrent administration of RLF

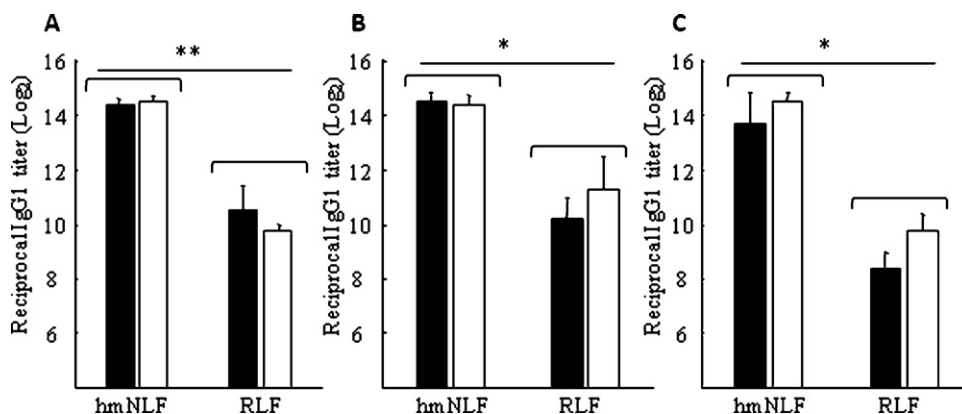


Fig. 1. Differential antibody induction by native human milk and rice recombinant lactoferrins. Mice ($n = 10$ per experiment) received 1% hmNLF or 1% RLF by intraperitoneal injection on days 0 and 7. Fourteen days after initiation of exposure, individual serum samples were prepared and analysed for specific IgG1 antibody by ELISA. Titre was determined as the maximum dilution of serum at which an OD450 nm reading of greater than 0.5 was achieved. Data are displayed as the mean $\pm$ SEM of reciprocal IgG1 antibody titres ($\log_2$) (3 independent experiments A, B, C) against homologous (■) and heterologous (□) substrates. Significant differences between hmNLF vs RLF treatment groups are indicated (one way ANOVA, followed by Bonferroni post hoc test; ** $p < 0.001$; * $p < 0.01$). There were no significant differences between homologous and heterologous substrates for each immunisation group. Negative control serum samples (from naïve animals) were tested concurrently and were routinely negative (data not shown).

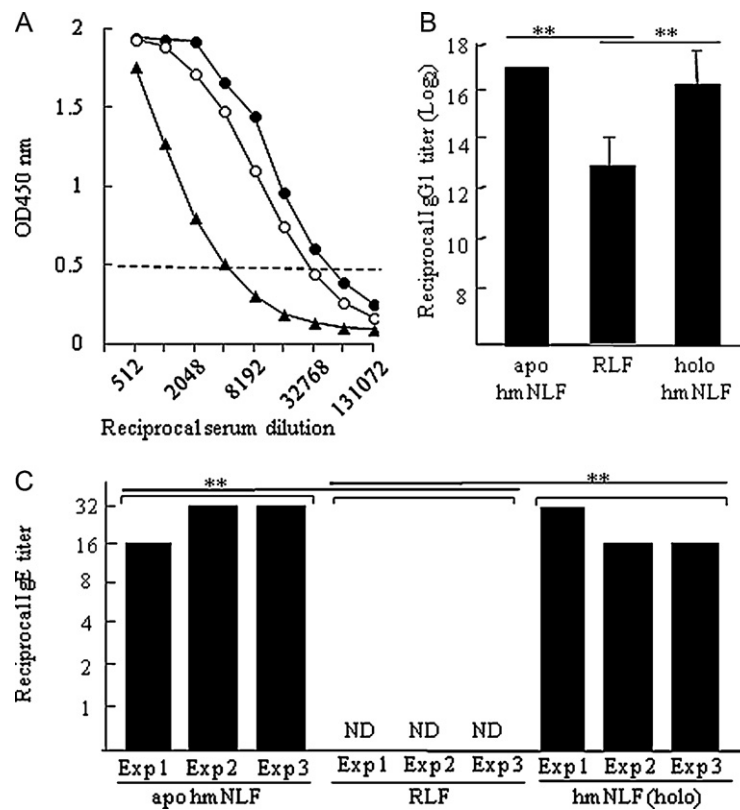


Fig. 2. The effect of iron saturation on antibody responses to lactoferrin. Mice ($n=10$) received 1% hmNLF (either the apo or the holo form) or 1% RLF by intraperitoneal injection on days 0 and 7. Fourteen days after initiation of exposure, individual and pooled serum samples were prepared and analysed for specific IgG1 antibody by ELISA. Reciprocal titration curves are shown for pooled serum samples from mice exposed to 1% hmNLF in the apo (●) or holo (○) forms or to RLF (▲) (A). The horizontal line denotes the cut off for positive responses (OD450 nm reading of 0.5). Individual serum samples were analysed for IgG1 (B). Titre was determined as the maximum dilution of serum at which an OD450 nm reading of >0.5 was achieved and expressed as mean (and SEM) IgG1 reciprocal titre ($\log_2$). Pooled serum samples were analysed for IgE by PCA assay for 3 independent experiments displayed as reciprocal IgE titres (C). ND = no detectable IgE in neat serum (IgE negative). Significant differences between hmNLF apo and holo forms vs RLF are shown (one way ANOVA, followed by Bonferroni post hoc test; $**p < 0.001$). Negative control serum samples (from naïve animals) were tested concurrently and were routinely negative in both the ELISA and PCA (data not shown).

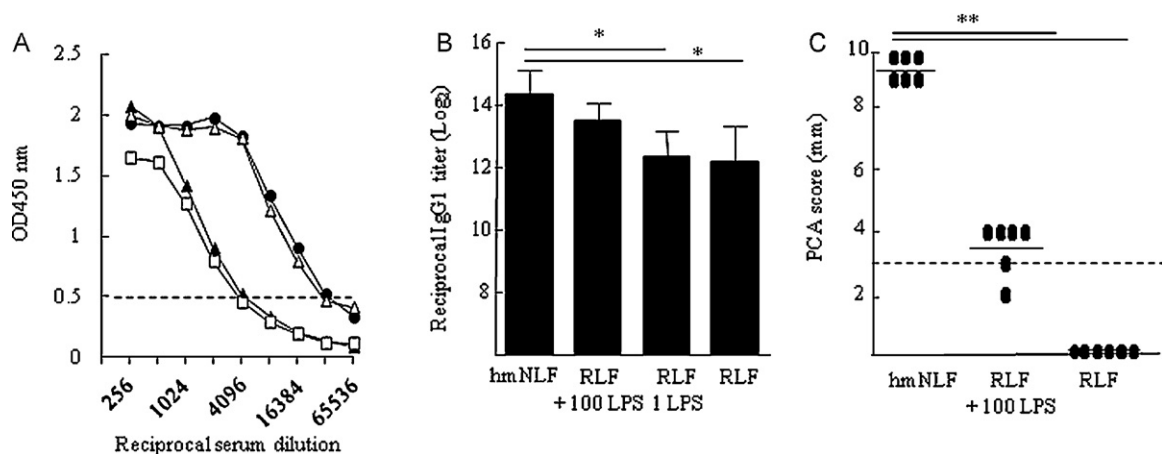


Fig. 3. The effect of LPS on antibody responses to lactoferrin. Mice ($n=6$) received 1% hmNLF or 1% RLF in the presence or absence of 1 or 100 µg/ml exogenous O55:B55 LPS (*E. coli*) by intraperitoneal injection on days 0 and 7. Fourteen days after initiation of exposure, individual and pooled serum samples were prepared and analysed for specific IgG1 antibody by ELISA. Reciprocal titration curves are shown for pooled serum samples from mice exposed to 1% hmNLF (●) or to RLF alone (▲) or in the presence of 1 (□) or 100 (Δ) µg/ml LPS (A). The horizontal line denotes the cut off for positive responses (OD450 nm reading of 0.5). Individual serum samples were analysed for IgG1 (B). Titre was determined as the maximum dilution of serum at which an OD450 nm reading of >0.5 was achieved and are expressed as mean (and SEM) IgG1 reciprocal titre ($\log_2$). Individual serum samples were analysed for IgE responders by PCA assay (C). (●) represents the mean PCA score of an individual neat serum sample tested in $n=2$ animals and each line represents the mean PCA score per treatment group. The horizontal line denotes the cut off for positive responses for IgE antibody (3 mm). Significant differences between IgG1 titres for hmNLF vs RLF alone and in the presence of 1 or 100 µg of LPS are shown (one way ANOVA, followed by Bonferroni post hoc test; $*p < 0.01$; B). For IgE responders, statistical significance of differences between groups was assessed by Fishers' exact test ($**p < 0.001$). Negative control serum samples (from naïve animals) were tested concurrently and were routinely negative in both the ELISA and PCA (data not shown).

with 1 μg LPS was without effect on the titration curve, but in the presence of 100 μg LPS, the titration curve shifted markedly to the right such that it was very similar to that recorded for hmNLF. The influence of LPS was confirmed by analysis of IgG1 antibody titres for individual serum samples (Fig. 3B), with the 100 μg dose of LPS inducing significant increases in IgG1 antibody responses that were equivalent to those provoked by hmNLF alone. Whereas the 1 μg dose was without significant effect on IgG1 antibody production. Despite the vigorous adjuvant effect of the high dose LPS on serum IgG1 antibody responses, the impact on IgE antibody production was considerably less pronounced. The number of IgE responders following administration of hmNLF and RLF in the presence and absence of 100 μg LPS was analysed by PCA (Fig. 3C). All mice (6/6) were strongly IgE positive following immunisation with hmNLF. Mice treated with RLF alone were uniformly negative for specific IgE, with 0/6 IgE responders recorded. High dose LPS did enhance IgE responses to RLF, with the majority (5/6) samples testing positive for IgE, however, such were somewhat borderline (mean PCA score 3.5 mm). These differences were reflected in the IgE antibody titres, with RLF alone and in the presence of 1 μg LPS failing to provoke detectable IgE antibody (titre <1) whereas in the presence of 100 μg LPS a titre of 1 in 8 was achieved, considerably lower than the titre of 1 in 32 recorded following immunisation with NLF.

3.3. Native and rice recombinant LF are equally labile to digestion with pepsin

Another factor that has been suggested to be an important characteristic of allergens is stability to digestion with pepsin (Astwood et al., 1996; Huby et al., 2000). The stability of both hmNLF and RLF and a control allergenic protein from cows' milk, βLG (shown previously to be pepsin resistant (Astwood et al., 1996; Thomas et al., 2004)) to digestion in SGF was investigated (Thomas et al., 2004). Following pepsin digestion, samples were analysed by SDS-PAGE. Coomassie staining of representative gels is displayed (Fig. 4). Incubation of protein and reaction buffer alone for 0 and 60 min resulted in a single clear band for all three proteins (lanes 1 and 2). These analyses demonstrated that both LF proteins and βLG were stable and did not undergo any self-digestion. Furthermore, the molecular weight of both forms of LF was similar; approximately 75 kDa. A band for pepsin at approximately 37 kDa was observed for all the digestion (protein plus pepsin) time points between 0 and 60 min on all three gels (lanes 3–10), as well as the pepsin alone controls sampled at 0 and 60 min (lanes 11 and 12). This confirmed the presence of pepsin in all digestion samples and the lack of pepsin auto digestion over the time course. Digestion of hmNLF (Fig. 4A) and RLF (Fig. 4B) proteins by pepsin in SGF followed very similar time courses. In both cases the protein was very labile. This was confirmed by densitometric analysis showing that by 2 min both proteins were completely undetectable (data not shown). No detectable fragments were observed following digestion. βLG was very resistant to digestion with pepsin, with a clear protein band present throughout the time course (Fig. 4C) and densitometric analyses revealing that only after 30 and 60 min was there any appreciable loss of protein (40% digestion at these later time points).

3.4. Comparisons of the immunogenicity and allergenicity of human milk and neutrophil NLF

Taken together, the data suggest that differential glycosylation patterns of the different forms of LF are responsible for its ability to provoke differential antibody responses *in vivo*. One of the main differences between the two molecules is the expression of Le^x trisaccharides by hmNLF. There is available commercially

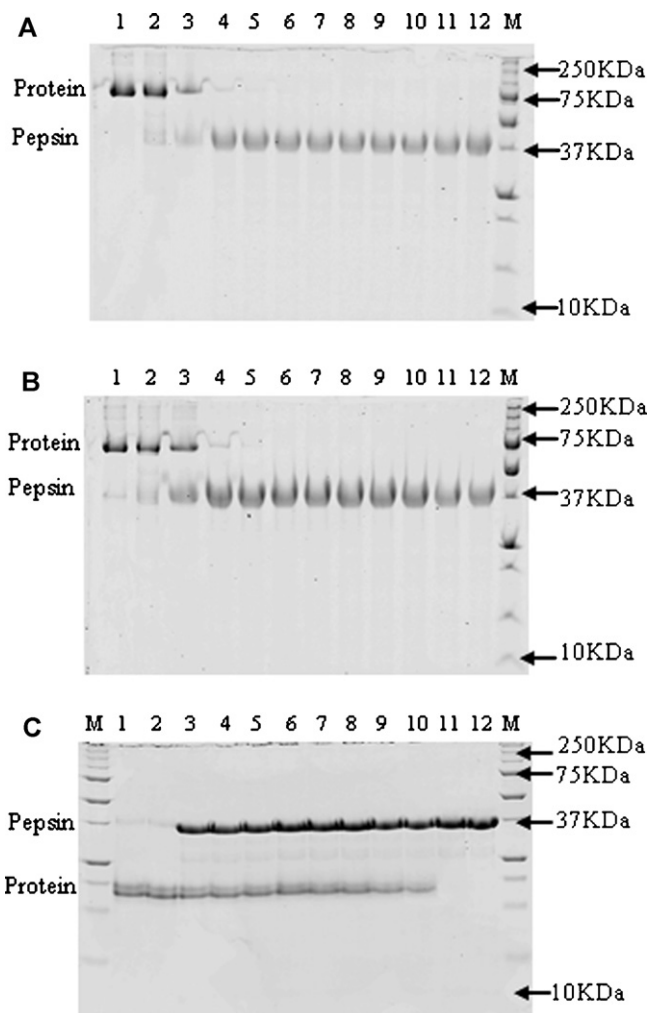


Fig. 4. Stability of lactoferrin to pepsin digestion. Protein preparations (hmNLF, RLF or βLG) were each incubated with simulated gastric fluid for 60 min at 37 °C. At 0.5, 2, 5, 10, 20, 30 and 60 min samples were removed and the reaction quenched. Controls were prepared concurrently and quenched at 0 and 60 min (test protein alone in reaction buffer to show stability of the test protein in the absence of pepsin; pepsin and no test protein to show autodigestion of pepsin). The samples were analysed by SDS-PAGE (10% Tris-HCl gel) and stained with Coomassie brilliant blue. Gels for hmNLF (A), RLF (B) and βLG (C) are shown. Molecular weight markers are indicated (M), with molecular weights ranging from 10 to 250 kDa. Lanes 1 and 2 are protein alone incubated for 0 and 60 min, lanes 3–10 are protein incubated with pepsin for 0, 0.5, 2, 5, 10, 20, 30 and 60 min, lanes 11 and 12 are pepsin alone incubated for 0 and 60 min.

another form of LF with even higher levels of Le^x than those found in the hmNLF: LF isolated from human neutrophil granules (nNLF) (Graham et al., 2011). Thus, all nNLF molecules carry clusters of Le^x epitopes whereas some molecules of hmNLF may only carry one molecule (Graham et al., 2011). The neutrophil protein displayed a similar lack of resistance to pepsin digestion to the other LF proteins (data not shown). In order to determine whether increased Le^x expression impacted on the antibody responses to LF, mice were exposed to hmNLF or nNLF at 0.01% (w/v) and antigen specific IgG1 (Fig. 5A) and IgE (Fig. 5B) antibody responses measured. Under these conditions of exposure, both materials induced detectable, albeit weak, IgG1 antibody responses, although hmNLF provoked significantly higher levels of IgG1 antibody than did nNLF ($p < 0.01$). Interestingly, under conditions where IgG1 antibody responses were significantly weaker, the neutrophil derived NLF provoked somewhat more vigorous IgE antibody responses than did the hmNLF.

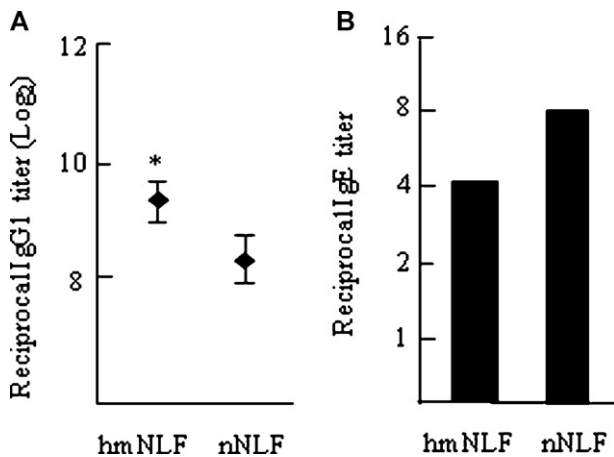


Fig. 5. IgG and IgE antibody responses to lactoferrin isolated from human neutrophils. Mice ($n=5$) received 0.01% hmNLF or NLF from neutrophils (nNLF) by intraperitoneal injection on days 0 and 7. Fourteen days after initiation of exposure, individual serum samples were prepared and analysed for specific IgG1 (A) antibody by ELISA and pooled serum samples for IgE antibody by PCA assay (B). Data are displayed as mean (and SEM) IgG1 reciprocal titre ($\log_2$) and IgE reciprocal titre. Significant differences between hmNLF vs nNLF treatment groups were analysed by Students' t test ($*p < 0.01$). Negative control serum samples (from naïve animals) were tested concurrently and were routinely negative in both the ELISA and PCA assay (data not shown).

4. Discussion

Exposure of BALB/c strain mice to NLF (isolated from either human milk or from neutrophils) stimulated vigorous IgG1 and IgE antibody responses whereas the recombinant protein raised in rice with an identical amino acid sequence was considerably less immunogenic and failed to stimulate detectable IgE antibody production. It was also of interest that all three proteins were relatively labile to pepsin digestion, given that resistance to pepsin has been suggested to be associated with allergenicity (Astwood et al., 1996) and is currently one of the recommended tests used to assess potential allergenicity of recombinant proteins (Mondal et al., 2011). However, this relationship between allergenicity and stability is by no means complete, with examples of pepsin-labile allergens and of pepsin-stable non-allergens (Griesmeier et al., 2010; Thomas et al., 2004). It may be that the positive predictability of pepsin digestibility is better than its negative predictability; thus it has been suggested that if >10% of the intact protein, or stable fragments >5 kDa, persist for 20 min or more, that the protein carries an increased risk of allergenicity (Ofori-Anti et al., 2008).

It was necessary to eliminate the possibility that these differential immune responses were due to biochemical differences between the LF preparations such as iron content or endotoxin contamination. These are legitimate potential confounding factors, given that there is evidence that some allergens have ion binding capacity, such as the fish allergen, parvalbumin, which binds calcium and IgE recognition is influenced by the calcium content of the protein (Wopfner et al., 2007). Furthermore, endotoxin (LPS) has been shown to have adjuvant effects in mice following dermal or inhalation exposure (Eisenbarth et al., 2002; Slater et al., 1998). IgE (and IgG subclass) antibody production by B cells is orchestrated by T helper (Th) cell subsets designated Th1 and Th2, with the balance between inhibitory cytokines such as interferon (IFN)- γ from Th1 cells and promotional cytokines such as interleukin (IL)-4 and IL-13 critical for determining whether conditions are permissive for IgE antibody production (Romagnani, 2000). Interestingly, the quality of immune response that is stimulated by LPS is apparently dose dependent, with lower doses augmenting Th2 type (IgG1

and IgE) and higher doses resulting in Th1 type (IgG2a) responses following administration through the respiratory tract (Eisenbarth et al., 2002). All LF preparations contained low levels of endogenous endotoxin contamination (<10 EU/mg protein). The addition of relatively low level LPS (1.2×10^3 EU) was without significant effect on IgG or IgE antibody responses; these levels are similar to levels of endogenous endotoxin found in some protein preparations (Dearman and Kimber, 2007). High dose LPS (1.2×10^5 EU) had a pronounced type 2 adjuvant effect on responses to RLF, with marked enhancement of IgG1 and IgE antibody responses. Despite the very high dose of endotoxin used, equivalent IgE titres to those stimulated by NLF were not achieved. It appears that antibody responses provoked by the intraperitoneal immunisation route are considerably less susceptible to the adjuvant effects of endotoxin, given that the low dose used in the current experiments is equivalent to that which was shown to enhance IgE when administered by inhalation (Eisenbarth et al., 2002).

It is proposed, therefore, that the divergent IgE (and IgG1) responses provoked by the different forms of LF are due to the differential patterns of glycosylation. Previous reports showing an impact of glycosylation on allergic responses have been largely confined to demonstrating that certain sugar residues contribute to IgE antibody binding sites (Dedic et al., 2009). In the studies reported herein, however, glycosylation was shown to be without effect on antibody binding. Thus, identical IgG and IgG1 titres were recorded regardless of the form of LF used as substrate in the ELISA for analysis of anti-hmNLF and anti-RLF anti-sera and anti-NLF IgE epitopes were detected on both hmNLF and RLF. The impact of the sugar residues was, therefore, at the level of induction of antibody production, presumably due to differential processing and presentation by antigen-presenting cells and/or induction of divergent T helper or T regulatory cell populations. The lack of immunogenicity (and allergenicity) of the RLF was associated with high levels of mannosylation, whereas the more vigorous immune responses of the NLF preparations were associated with high levels of Le^x residues (Almond et al., unpublished observations; Graham et al., 2011). Binding of preformed IgE has been shown to be influenced by the presence of mannose residues. Thus, plant (*Arabidopsis*) proteins expressed by mutants lacking Golgi α -mannosidase II carry β 1,2-xylose and α 1,3-fucose epitopes that are masked by mannose residues. IgE containing sera from allergic donors directed against xylose-containing N-glycans (linked through asparagines) displayed reduced binding that could be restored by glycosidase treatment (Kaufmüller-Soboll et al., 2011b). However, there is little published information available regarding the role of mannosylation on the induction of antibody responses to allergens (and IgE antibody production). There is a more substantial literature supporting the impact of these sugar residues on immunogenicity (IgG antibody and T cell responses). Production of recombinant mannosylated ovalbumin (a model antigen and allergen) in yeast revealed enhanced immunogenicity with respect to CD4 and CD8 T cell proliferative responses, although antibody responses were not measured (Luong et al., 2007). Furthermore, the nature of the mannosylation was critical, with O-linked mannose residues (linked through serine/threonine) associated with increased lymphoproliferative responses whereas N-linked mannosylation resulted in decreased responses. There are other reports that mannosylation can decrease immunogenicity, although such was suggested to be related to masking of epitopes by the sugar molecules. Thus, mutant versions of β LG with high mannose content produced in yeast *Pichia pastoris* elicited weaker antibody responses in BALB/c, C57BL/6, and C3H/He mice than did wild type β LG (Tatsumi et al., 2012). In summary, the evidence suggests that mannosylated proteins, particularly N-linked mannoses, are less immunogenic (and therefore likely to be less allergenic) than non-mannosylated proteins.

The allergenic forms of LF were associated with Le^x residues, with more vigorous IgE responses recorded for nNLF compared with hmNLF. Human milk NLF contains both Le^x and Le^y groups, whereas nNLF only contains Le^x motifs and uniformly expresses clusters of Le^x epitopes whereas some molecules of hmNLF may only carry one molecule at most (Graham et al., 2011). The Le^x residues have been associated previously with IgE antibody responses (Harn et al., 2009; Hsu et al., 2007). Parasitic infections modulate host immune responses, and Le^x motifs have been shown to be one of key immunomodulatory glycans important in the induction of Th2-type immune responses (Harn et al., 2009). Furthermore, the synthetic trisaccharide molecule Le^x was able to act as an adjuvant for specific IgE responses induced by the reference allergen ovalbumin, although such was less effective than conjugation of the sugar to the protein (Hsu et al., 2007). Although the mechanism is not elucidated fully, it has also been shown that multiple copy Le^x conjugates are able to activate antigen-presenting cells, acting through C-type lectin binding (Harn et al., 2009) and inhibition of IL-12 production; a cytokine that promotes Th1 and inhibits Th2 activity (Hsu et al., 2007). Interestingly, coadministration of mannan, a polymer of mannose, reversed the suppressive effect of Le^x trisaccharides on IL-12 production, suggesting reciprocal effects of these sugars as recorded herein.

In addition to the implications for the role of glycosylation in potential allergenicity of GM crop plants, the issue is also of relevance to therapeutic proteins. More than 60% of current therapeutic proteins are glycosylated, and given the influence of this posttranslational modification on various pharmacological properties such as stability, and pharmacokinetics, there is much interest in targeting this process to improve the properties of new or licensed products (Li and d'Anjou, 2009). Differential glycosylation may expose immunogenic epitopes, given that recombinant material produced in *E. coli* and lacking glycosylation showed increased immunogenicity in patients (Gribben et al., 1990). Other carbohydrate moieties may represent IgE epitopes, as was discovered recently following treatment of patients with metastatic colorectal cancer and squamous-cell carcinoma of the head and neck with the mouse–human chimeric antibody cetuximab. This antibody expresses a carbohydrate galactose- α -1,3-galactose that is found commonly on nonprimate mammalian proteins which was shown to be responsible for eliciting serious, even fatal, anaphylactic reactions (Chung et al., 2008). The majority of the patients were shown to express pre-existing IgE antibody to this moiety and it has subsequently been shown to be an important, and hitherto unrecognised, epitope in individuals that suffer severe IgE-mediated responses to red meats (beef, pork, lamb) (Commins et al., 2009).

In summary, these data demonstrate that differential glycosylation profiles can have a profound impact on protein allergenicity and immunogenicity, with mannose and Le^x exhibiting opposing effects on IgE antibody responses. These results have potential relevance for understanding the potential allergenicity of novel proteins in GM crops as well as implications for the design of therapeutic proteins with improved characteristics with respect to immunogenicity.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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