Toxicology 301 (2012) 50–57

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

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

Rachael J. Almond[a,*], Brian F. Flanagan[b], Ian Kimber[a], Rebecca J. Dearman[a]

[a] Faculty of Life Sciences, The University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK
[b] Department of Clinical Microbiology and Immunology, Institute of Global Health, Duncan Building, Daulby Street, Liverpool L69 3GA, UK

ARTICLE INFO

Article history:
Received 16 April 2012
Received in revised form 11 June 2012
Accepted 25 June 2012
Available online 17 July 2012

Keywords:
Protein allergy
Immunotoxicology
Glycosylation
GMO crops
Expression systems
Safety assessment

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.

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* (ovamocoid 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

* Corresponding author. Tel.: +44 161 2755368; fax: +44 161 2755586.
E-mail address: rachael.almond@manchester.ac.uk (R.J. Almond).

0300-483X/$ – see front matter
http://dx.doi.org/10.1016/j.tox.2012.06.018

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 ± 2 °C and relative humidity was 55 ± 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 βlactoglobulin from cows' milk (βLG; 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× 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 $NaHCO_3$ pH 11 and placed at >75 °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 immunosorbant assay (ELISA) as described previously (Dearman et al., 2003). Plastic microtitre plates (Nunc, Copenhagen, Denmark) were coated with 100 μg/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 peroxidise 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 $\log_2$ 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 ($\log_2$). 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).