The Dalton size debate in extensively hydrolysed formulas

By Rosan Meyer, Paediatric Dietitian
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Introduction

It is thought that cows’ milk allergy (CMA) affects 2-4% of children worldwide. The mainstay of management remains the complete elimination of cows’ milk (CM), including its derivatives from the diet1. Breastmilk also continues to be the gold standard source of nutrition in children with CMA, however, when breastmilk is not available, current guidance suggests the use of a hypoallergenic formula as an alternative2. In 1999 the European Society for Paediatric Allergology and Clinical Immunology [now called the European Academy for Allergy and Clinical Immunology (EAACI)] and the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) suggested that products labelled with reduced allergenicity should comply with either of the following guidelines: an in vitro content of < 1% immunoreactive protein of total nitrogen containing substances, or that at least 90% of children with a proven CMA tolerate the feed with a 95% confidence interval3. According to this definition only extensively hydrolysed formula (EHF) or amino acid feeds are suitable for the management of CMA4-5. The only truly non-allergenic formula currently is an amino acid formula however, this has a significant cost implication for use and has in most guidelines been reserved for the more severe spectrum of CMA6,7. The majority of cows’ milk allergic conditions are therefore managed with EHFs. Previously the choices of EHFs were limited to one or two EHFs based on either casein (EHF-C) or whey (EHF-W). However, in recent years several new products have been launched, with an increase in claims of improved allergenicity based on peptide length or Dalton size. This has led to the re-emergence of discussion around the meaning of these parameters in cows’ milk allergic infants and whether this needs to be taken into account with the choice of formula.

In recent years several new products have been launched, with an increase in claims of improved allergenicity based on peptide length or Dalton size. This has led to the re-emergence of discussion around the meaning of these parameters in cows’ milk allergic infants and whether this needs to be taken into account with the choice of formula.

Determining the allergenicity of an EHF

In order to establish the allergenicity of EHFs, it is important to understand the protein composition of cows’ milk and also the process of producing a hydrolysed formula (Table 1). Cows’ milk contains both casein and whey proteins, with each of these fractions containing several allergenic proteins. These proteins contain epitopes that are divided into two categories which are classified according to their specific amino acid sequence; these can either be conformational or linear/sequential epitopes. For an allergic reaction to occur, circulating antibodies recognise specific conformational and/or linear epitopes on the antigen surface, which in turn leads to a cascade of immune reactions resulting in the symptoms associated with an allergic reaction to CM.

In order therefore to produce a hydrolysed formula suitable for the management of CMA, casein or whey proteins need to be hydrolysed (breaking of peptide bonds) in such a way that the recognition of these epitopes does not occur.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dalton size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-lactalbumin</td>
<td>14.2</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>160.0</td>
</tr>
<tr>
<td>Lactoferrin (trace)</td>
<td>80.0</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>18.2</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>67.0</td>
</tr>
<tr>
<td>αs1-casein</td>
<td>23.6</td>
</tr>
<tr>
<td>β-casein</td>
<td>25.2</td>
</tr>
<tr>
<td>β-lactalbumin</td>
<td>24.0</td>
</tr>
<tr>
<td>γ-casein</td>
<td>20.6</td>
</tr>
<tr>
<td>K-casein</td>
<td>11.8</td>
</tr>
<tr>
<td>X-casein</td>
<td>11.6</td>
</tr>
<tr>
<td>K-casein</td>
<td>19.0</td>
</tr>
</tbody>
</table>

Table 1: Protein in cow’s milk and Dalton size of each protein10

Hydrolysed formulas are processed using four main technologies to reduce the molecular weight of CMA:

1. Heat treatment - which mainly affects the conformational epitopes and leads to a reduction in allergenicity in particular for whey proteins, but not casein9.
2. Enzymatic hydrolysis (using trypsin, chymotrypsin and papain) – enzymatic cleavage of polypeptide chains leads to the destruction of sequential epitopes and affects both casein and whey9,11.
3. Enzymatic hydrolysis under hydrostatic pressure – enzymatic cleavage under pressure has been shown to yield even shorter chain lengths than standard enzymatic hydrolysis9,12.

Both extensively hydrolysed casein and whey formulas exist and in the majority of modern hydrolysates a combination of the above methods are used (Figure 1).

* The exact definition of “most or majority of peptides < 1.5kDa or 1 kDa” remains elusive, as the in vitro threshold for eliciting an allergic reaction has not been established.

Step 1: Heat treatment

Step 2: Enzymatic hydrolysis (with/without hydrostatic pressure)

Step 3: Ultrafiltration

The evidence for the use of Dalton size to select EHFs for the management of CMA

Allergenic properties of EHF can be characterised by biochemical techniques, such as the spectrum of the peptide sizes/molecular weight or the ratio of α-amino nitrogen to total nitrogen. The allergenic properties may be tested in vitro by various immunologic methods including Immunoglobulin E (IgE) binding tests such as radioallergosorbent test (RAST), RAST-inhibition test, and enzyme-linked immunosorbent assay (ELISA), and in vivo by the skin prick test (SPT) and the gold standard method, the oral challenge tests8,9,11,12.

The molecular weight of proteins and peptides are expressed in Daltons (Da) or kilo Daltons (kDa) and the extent of hydrolysis of feeds are therefore specified in this unit. Guidelines have often defined EHFs as formulas “where most of the nitrogen is in the form of free amino acids and peptides < 1.5 kDa”. The recent British Society for Allergy and Clinical Immunology guidelines have also stated that feeds where the greatest percentage of peptides are under 1 kDa may be preferable. The hypoallergenicity of amino acids is undisputed, however the exact definition of “most or majority of peptides < 1.5kDa or 1 kDa” remains elusive, as the in vitro threshold for eliciting an allergic reaction has not been established.
In order to explore the origin of this arbitrary Dalton size cut-off, one has to study research that is now > 20 years old. In 1993 Siemsenma et al. studied the importance of peptide lengths of commercially available EHF's using sodium dodecyl sulfate polyacylamide gel electrophoresis (SDS-PAGE), which was at that time a relatively new method, and identified three generations of EHF:

- The first generation (casein based) were characterised by the majority of the protein being amino acids (70 mol%) and detectable peptides of up to 5-8 amino acids long.
- The second generation (whey based) typically had 40-60 mol% free amino acids and detectable peptides up to 10-12 amino acids long.
- The third generation (whey based) had < 20 mol% free amino acids and detectable peptides of up to 10-15 amino acids long.

This generation of EHF has been developed due to the poor palatability of bitter tasting casein peptides, which has reduced the acceptability in children. Significant amounts of peptides of molecular weights > 1.5 kDa were not detected in any of the above feeds, however, in some there was a residue of < 1% peptides with a molecular weight of 3 kDa. Still a residue of < 1% peptides with a molecular weight of 3 kDa. It is known that in IgE-mediated CMA around 10% of children continue to react to an EHF and up to 30% in non-IgE-mediated CMA. However, this may not only be related to peptide size, but also residual intact proteins (i.e. β-lactoglobulin), and polymers or aggregates formed during the production or reconstitution. More recent in vitro studies have therefore focused on protein components that may elicit an allergic reaction (the ability of protein components to bind pre-existing antibodies), using a combination of SDS-PAGE, native PAGE, immunoblotting, dot-immunobinding and ELISA. A study by Rosendal and Barkholt combined these methods and ranked the allergenic potential of six different EHF’s as follows: EHF-casein (Nutramigen, Pregestimil), followed by EHF-whey (Afare, Nutrilon, Aipami) Pepti and Pepti Plus (stage 2), Pepti Junior, Profylac and Pregomin. Similar results were found in a Swedish study, with the EHF based on casein having the least allergenic potential. However as with the Dalton size, none of the above in vitro studies can predict a clinical reaction in a child with a proven CMA.

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Since the publication of this study in 1993, EHF’s have for some reason been judged by this arbitrary cut-off of having the majority of peptides “below 1.5 kDa”. However, this in vitro cut-off does not predict the allergenicity of an EHF in clinical practice.

Several studies were performed in the nineties and one in 2001 to translate in vitro hypoallergenicity into actual reactivity in children with confirmed CMA. These found conflicting results, summarised in Table 2 and 3.

Positive SPT and specific IgE results in both EHF-C and EHF-W have been documented with a variety of different peptide lengths. Similarly, challenge procedures to these formulas also yielded positive results to a small number of children with both casein and whey hydrolysates, all with the majority of peptides < 1.5 kDa, however, with variation in the percentage of peptides < 1 kDa. In fact, anaphylaxis to a variety of EHF’s has been reported. Host et al. explored the importance of β-lactoglobulin in mothers consuming cows’ milk versus EHF’s. This group found that β-lactoglobulin can be detected in the breastmilk of 95% of lactating women at a level of 0.9–150 lg/l (median 4.2 lg/l). Similarly low amounts (0.84–14.5 lg/l) of residual β-lactoglobulin have been found in EHF’s. In the past, it was thought that β-lactoglobulin, a whey protein, was the most important protein related to CMA; however, it has since been shown that other proteins, such as the different caseins, are also involved in the aetiology of this allergy. There is a paucity of data on the residues of other proteins in EHF’s (i.e. α-casein or β-casein), but one can hypothesise that reactions to EHF’s could also occur as a result of protein other than β-lactoglobulin.

Selection of hypoallergenic formulas according to new research

The guidelines by Host et al. in 1999 already recognised that in vitro characterisation of peptide size and determination of allergenicity might be valuable for quality control of the products and assurance of batch to batch consistency as well as for labelling, but on the basis of current knowledge, such data do not predict the immunogenic or the allergenic effects of CMA in infants.

In addition, different techniques may be used to measure the peptide size, which includes electrophoresis or chromatography, which may yield different results for the same product. There has been, therefore, an international drive by both EAACI and the AAP that products marketed for the management of CMA should be tolerated by at least 90% (with 95% confidence) of infants with documented CMA. Furthermore, it is recommended that after a successful double-blind, placebo-controlled challenge the product should be tolerated in a controlled open challenge during a period of seven days. Although the percentage of peptides below this arbitrary cut-off of 1.5 kDa differ between the EHF’s, studies have indicated that they are still tolerated by 90% of children (95 CI) with proven CMA. It would therefore be erroneous to make a judgement on hypoallergenic food preferences based on the Dalton size alone.
Summary points

• A hypoallergenic formula needs to comply with the following two definitions: an in vitro content of < 1% immunoreactive protein of total nitrogen containing substances, or that at least 90% of children with a proven CMA tolerate the feed with a 95% confidence interval.

• There has been a drive by international bodies in allergy and immunology that hypoallergenic feeds should be tested in the target population and comply in particular with the second definition of tolerance.

• Both EHF-casein and EHF-whey formulas exist with a variety of peptide lengths (all with the majority < 1.5 kDa) complying with the suggested definition.

• Peptide length does not allow for the prediction of clinical reactivity.

• Other factors outside of peptide length may lead to reactions (i.e. residue of β-lactoglobulin).

• EHFs should be recommended not on their peptide length, but on the basis of clinical studies in CMA children.

Conclusion

Based on current data, in vitro assessment of peptide size is useful for quality control and the labelling of EHFs, but it is not a reliable marker to predict reactivity in children with a CMA. All products marketed for the management of CMA should therefore have their efficacy tested in a clinical setting, indicating tolerance in 90% of children with proven CMA.

Appendix of terms

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Dalton</td>
<td>This is the standard unit that is used for indicating mass on an atomic or molecular scale and is used to provide an indication of the peptide length in EHFs</td>
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<tr>
<td>Epitopes</td>
<td>A molecular region on the surface of an antigen capable of eliciting an immune response</td>
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<tr>
<td>Conformational epitopes</td>
<td>Epitope with a specific three-dimensional shape</td>
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<tr>
<td>Linear/sequential epitopes</td>
<td>Epitope with a linear sequence of amino acids</td>
</tr>
<tr>
<td>Radio-allergosorbent test</td>
<td>This test is a radioimmunoassay to detect specific IgE antibodies to suspected or known allergens for the purpose of guiding a diagnosis</td>
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<tr>
<td>Enzyme-linked immunosorbent assay</td>
<td>This is a biochemical test that uses antibodies and an enzyme-mediated colour change to detect an antigen</td>
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<tr>
<td>Mol %</td>
<td>Percentage of moles. 1 kDa has a molecular weight of 1 kilogram per mole of protein</td>
</tr>
<tr>
<td>Chromatography</td>
<td>Chromatography is a process in which a chemical mixture carried by a liquid or gas is separated into components as a result of differential distribution of the solutes as they flow around or over a stationary liquid or solid phase</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field</td>
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<tr>
<td>Immunoblotting</td>
<td>Analytical technique used to detect specific proteins in a sample of tissue homogenate or extract (i.e. Western blot). It uses gel electrophoresis</td>
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</table>
References

20. Halken S, Host A. Hypoallergenic formulas—when, to whom and how long: after more than 15 years we know the right indication! Allergy 2004;59(Suppl)78:45-52.