

ORIGINAL PAPER

A hydrolysed rice-based formula is tolerated by children with cow's milk allergy: a multi-centre study

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Clinical and Experimental Allergy

Summary

Background Children allergic to cow's milk are fed a soy- or a hydrolysed cow's milk-based substitute. Neither can rule out a sensitization risk. Previous studies have shown that hydrolysed rice is tolerated by animals and children with multiple food hypersensitivities. **Objective** A prospective clinical assessment of tolerance to a rice-based hydrolysed formula was carried out in children allergic to cow's milk.

Patients and methods One hundred children (42 girls and 58 boys, mean age 3.17 ± 2.93 years, median 2.20, range 0.18–14.6 years) with a history of immediate reactions to cow's milk and confirmed at double-blind, placebo-controlled food challenge (DBPCFC) when not contraindicated were assessed for clinical tolerance to cow's milk proteins. Their allergy work-up included skin prick tests with whole milk, α -lactalbumin (ALA), β -lactoglobulin (BLG) and total caseins, and specific IgE determinations using CAP technology were performed against whole milk, ALA, BLG and casein. Sensitization to rice and rice-based hydrolysed formula was similarly investigated. Patients' sera were evaluated at immunoblotting for specific IgE to cow's milk proteins, rice and rice-based hydrolysed formula. DBPCFC was carried out with increasing doses of a rice-based hydrolysed formula. **Results** All patients were sensitized to cow's milk and/or at least one cow's milk protein fraction. Eighty-seven out of 99 were positive to cow's milk and/or a cow's milk protein fraction at skin prick test. Positive (> 0.35 kUA/L) specific IgE determinations were found for cow's milk and/or milk fractions (92/95), rice (21/91) and hydrolysed rice infant formula (4/91). At immunoblotting, sera from 96 children were positive to α -casein ($n = 54$), β -casein ($n = 38$), ALA ($n = 57$), BLG ($n = 37$) and bovine serum albumin ($n = 61$). Similarly, although patients' sera often contained specific IgE against rice proteins at CAP (21/91) and immunoblotting (70/96), only six very weakly positive responses were observed against rice-based hydrolysed formula. All DBPCFC with rice-based hydrolysed formula were negative.

Conclusions Rice-based hydrolysed formula is a possible alternative not only for children with multiple allergies, but also for children with cow's milk allergy.

Keywords food allergy, hydrolysed formula, infant formula, milk hypersensitivity, rice hydrolysate, tolerance

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Introduction

The management of infants and children with confirmed IgE-mediated cow's milk allergy (CMA) leaves the paedia-

trician with several dietary options, none of which, given the prevalence, spectrum and potential seriousness of the condition [1–5], can be recommended to all patients with CMA. In the absence of an alternative to cow's milk, the

management of CMA should use safe, affordable and nutritionally adequate preparations in line with European Society for Paediatric Gastroenterology, Hepatology and Nutrition standards [6–9]. Extensively hydrolysed cow's milk protein (eHF), which are considered as safe for most children with CMA, are still liable to contain residual peptides, and hypersensitivity reactions may thus occur in infants allergic to cow's milk [10]. Thus, specific product allergenicity and benefits must be addressed on an individual basis prior to recommending a hydrolysate as a substitute for cow's milk [11]. Soy formulae can also sensitize CMA children to soy [12–16], and use of soy hydrolysates is supported by few dietary intervention studies [17]. Elemental amino-acid-based formulae have been studied from safety and nutritional efficacy perspectives [18, 19], but their widespread recommendation remains limited owing to economic and palatability considerations [20]. The alternative, that of not substituting milk at all, presents obvious nutritional drawbacks [21]. Balancing these options and their pitfalls against variables such as efficacy, adequate nutrient intake, patient preferences and costs, consensus documents suggest that either a soy formula [22] or cow's milk hydrolysates [23] should be the first alternative to cow's milk for children with CMA. However, in a cohort of 170 patients with CMA followed from infancy up to their second year, adverse reactions at double-blind, placebo-controlled food challenge (DBPCFC) to soy (10%) and eHF (2.2%) have been reported in children fed these substitute formulae [15]. A more positive outcome was achieved with a hydrolysed rice infant formula (HRF) hypoallergenic in animals [24], which was well tolerated by children with multiple food allergies [25]. In a pilot study, data suggest that HRF was nutritionally valid [26]. A series of clinical and management questions regarding HRF use remain unanswered. Children with CMA can be sensitized to rice, but levels of specific IgE to HRF have been left unassessed [24]. How many children with CMA and without soy allergy are sensitized or allergic to rice is not known, nor whether sensitization to rice is because of cross-sensitization with soy. Another concern is that only one study has assessed the clinical tolerance of HRF in children [25]. Without larger studies of children with CMA and without multiple allergies, a comparison between HRF, soy formulae and eHFs may not be carried out. Against this background and unmet needs, we prospectively assessed the clinical tolerance of HRF to investigate its safety and potential as a substitute formula for children with immediate reactions to cow's milk.

Patients and methods

Between 1st January, 2003 and 30th April, 2004, 100 patients were recruited from nine hospitals in Bolzano, Florence, Messina, Milan, Naples, Pavia, Pescara and Rome

(two centres). Eligibility was limited to patients with immediate reactions confirmed by positive cow's milk DBPCFC or anaphylaxis in the 2 months prior to enrollment [27]. Children with soy allergy were excluded. Ethical approval from the nine Internal Review Boards and informed parental consent was sought prior to enrollment.

Hydrolysed rice infant formula

HRF (RisolacTM, Heinz Italia, Plasmon, Milan) was used. The protein source is an extensively hydrolysed rice protein isolate, supplemented with L-lysine and L-threonine in order to bring amino acid composition closer to that of human milk. The protein efficiency ratio (as a standard for evaluating the HRF protein quality) was equal to 106% of casein (data not shown). The complete nutritional and molecular weight profiles of the HRF have been reported elsewhere [25].

Milk proteins

Highly purified cow's milk proteins (Sigma Chemical, St Louis, MO, USA) were used in the preparation and carrying out of skin prick test (SPT) material and immunoblottings.

Skin tests

SPT were performed by percutaneous lancing through a drop of allergen extract immediately wiped off with absorbent paper using 1 mm-tipped lancets (Dome-Hollister-Stier, Warwickshire, UK). A solution of histamine phosphate in 50% glycosaline (10 mg/mL) and the vehicle were used as positive and negative controls, respectively. Weal diameters were read through a clear plastic caliper disk scaled in quarter-millimetres under $\times 4$ magnification and recorded as positive when a ≥ 3 mm weal margin (SPT cut-off point) was included within a complete caliper circle, rounding up/down measurements to the nearest millimetre [28]. To minimize investigator bias clinical guarantors at each participating centre were trained during a pre-trial investigator meeting in December 2002. Rice, whole milk, α -lactalbumin (ALA), β -lactoglobulin (BLG) and total casein (Cas) allergens were solubilized in the vehicle at the 5% final protein concentration. The vehicle, containing 2% mannitol, 5.9% NaCl, 58% glycerol and 0.4% phenol, was sterilized by microfiltration. The rice-based hydrolysed formula (RisolacTM, Heinz-Plada, Italy) was suspended in the vehicle to obtain a 5% final protein concentration and was then diluted with the vehicle: 1 : 10, 1 : 100, 1 : 1000 (v : v). Fresh food SPT was also performed with fresh pasteurized cow's milk.

Serology

Food-specific IgE for cow's milk, single CM proteins (ALA, BLG, Cas), rice and HRF were determined by processing

patients' sera from venous blood samples obtained at the time of enrolment with the automated Pharmacia CAP system FEIA from Pharmacia & Upjohn Diagnostics (Uppsala, Sweden), and the cut-off point for positivity was set at 0.35 kUA/L. CAP devices for HRF were prepared specifically for this study (Pharmacia). All sera were sent to Milan and CAP determinations were performed at the Melloni Hospital.

Sodium dodecyl sulfate electrophoresis and immunoblotting.

Samples were separated on a gradient polyacrylamide gel with the following characteristics:

Gradient running gel: 9–19% acrylamide; 0.08–0.17% bis-acrylamide; 0.36 M TRIS-HCl buffer pH 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% *N,N,N',N'*-tetramethylethylenediamine (TEMED).

Stacking gel: 3.5% acrylamide; 0.09% bis-acrylamide; 0.125 M Tris-HCl buffer pH 6.8; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% (TEMED).

Running buffer: 25 mM TRIS, 0.19 M glycine and 0.1% SDS (w/v), pH 8.8.

After the electrophoretic run (90 V at room temperature, for approximately 6 h), gels were dyed with Coomassie Brilliant Blue G-250 by the method of Neuhoof et al. [29]. All materials and instruments were purchased from Bio-Rad (Richmond CA, USA).

After SDS-PAGE, proteins were transferred onto a PVDF membrane (Millipore) by Western blotting in a Trans-blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin solution (in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.055 Triton-X) to prevent non-specific adsorption of the immunological reagents. The membrane was then immersed in 10 mL of 0.25% gelatin solution, containing 0.3 mL of serum from allergic children. Antigen-IgE complex was detected using goat anti-human IgE antibodies labelled with alkaline phosphatase; finally, after incubation in the bromo-chloro-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) solution, an intense precipitate developed at the site of the enzyme binding. Quantitative evaluation of the immunoreactive bands was performed by a gel scanner (Sharp JX-330, Pharmacia Biothec) and the Image Master™ 1D Software. It allows the quantification of proteins by calculating the average density of pixels across the band length and integrating over the bandwidth.

Classes of positive reactions were defined on the basis of an arbitrary scale of densitometric values, and six classes of reactivity were identified.

Purified cow's milk proteins, rice and HRF were prepared in sample buffer at a final protein concentration of 1 mg/mL; sample buffer contained 0.125 M Tris-HCl pH 6.8, 3.85 glycerol, 1% SDS and 2.5% mercaptoethanol.

Oral provocation tests

DBPCFC procedures were carried out according to the methods outlined elsewhere [25]. Briefly, doubling doses of 12, 24, 48 and 96 mL fresh pasteurized cow's milk mixed with placebo (90+90 mL) were administered unless symptoms developed. Neocate™ was used as placebo after negative skin test. Blinded preparations (Neocate™ or HRF masked in Neocate™ formula) were used in DBPCFC on 2 separate days. The HRF concentration (12.5 g/100 mL) was based on the highest manufacturer-recommended dose (13 g/100 mL) and was masked in Neocate™ (1:1 w:w). The placebo was made up of 25 g/100 mL Neocate™. Challenge dose increments for both preparations were: 0.2, 0.6, 2.0, 6.0, 20.0, 60.0 and 200.0 mL, corresponding to 0.025, 0.075, 0.25, 0.75, 2.5, 7.5 and 25 g of the original rice formula. Both were administered at intervals of 30 min.

Results

One hundred children (42 girls, 58 boys; mean age 3.17 ± 2.93 years; median age 2.20; range 0.18–14.6 years) were recruited. As patients with a history of anaphylaxis should not be challenged, cow's milk allergy could be confirmed by DBPCFC in 84/100 children. Clinical symptoms are shown in Table 1. All participants (except for one child with severe eczema) underwent SPT as part of the protocol. At enrollment, 16 patients were exclusively breastfed infants and other participants were receiving soy formula ($n = 32$), cow's milk hydrolysates ($n = 15$), an amino acid mixture ($n = 2$) and soy hydrolysate ($n = 6$). Three children followed the Rezza-Cardi diet, a home-prepared, lamb-based regimen [30]. All children consumed rice, except six, who were not yet consuming solid foods. With the cut-off point for positivity set at SPT by a weal diameter larger than 3 mm, 87 patients were found as being sensitized to cow's milk or one of its proteins, with casein, ALA and BLG being the three main sensitizing allergens (Table 2). Specific IgE to milk were

Table 1. Symptoms at enrollment of children with CMA confirmed at history or by oral provocation test

Symptom	N
Anaphylaxis	16*
Urticaria	32
Asthma	26
Gastrointestinal	24
Angio-oedema	11
Rhinitis	9
Laryngospasm	1

*Untested at DBPCFC

CMA, Cow's milk allergy; DBPCFC, double-blind, placebo-controlled food challenge.

measured in 93 patients as three sera were lost during transfer to Milan and some samples from the youngest infants were not sufficient to perform all tests. IgE determinations are reported in Table 2. All children included on the grounds of a history of anaphylaxis returned positive SPT and CAP test results with cow's milk. Positive responses in immunoblotting were mainly associated with bovine serum albumin, α -casein, β -casein and ALA. Responses to rice were confined to low (class 1) or moderate (class 2) reactivity. However, 90/96 children returned negative immunoblottings with HRF. Even when

highly positive to rice (classes 3 and 4), responses were mostly negative to HRF (Fig. 1). When positive, the reactions to HRF belonged to class 1, that is to say, the lowest class considered significant. SPT with rice was positive in only four children, three of whom were also positive to HRF 5%. Only one patient responded to SPT at 1:10 concentration and none to dilutions equal to or superior than 1:100 v:v. There were three discordant results between SPT and specific IgE determinations for cow's milk or their individual proteins. Thirteen children were positive at serology and/or immunoblotting evaluations but negative at SPT. All patients for whom it had been prescribed were able to undergo DBPCFC and no procedure had to be interrupted. All challenges with HRF or placebo were uneventful.

Table 2. Sensitization indices in 100 children with CMA

	SPT+	CAP+	Immunoblotting+
CM	85/99*	80/93 [†]	92/96 [†]
ALA	72/99	61/91	57/96
BLG	73/99	61/91	20/96
α -Cas			54/96
β -Cas	74/99	63/91	38/96
BSA			61/96
Rice	4/99	21/91	70/96
HRF	3/99	4/91	6/96

*One subject was excluded because of severe eczema.

[†]Lost or insufficient sample to perform all assays.

CMA, Cow's milk allergy; SPT, skin prick test; ALA, α -lactalbumin; BLG, β -lactoglobulin; BSA, bovine serum albumin; HRF, hydrolysed rice infant formula; Cas, casein.

Discussion

This is the first study to assess the tolerance of an HRF in a population of infants and children with immediate hypersensitivity to cow's milk. We did not focus on infants alone nor did we plan a comparative, interventional or natural history study. Rather, ours is a clinical tolerance study of an alternative protein source for children with immediate reactions to cow's milk, irrespective of what other sensitization they may have, in order to widen application of the results beyond subgroups of selected patients. The pattern of sensitization observed confirms

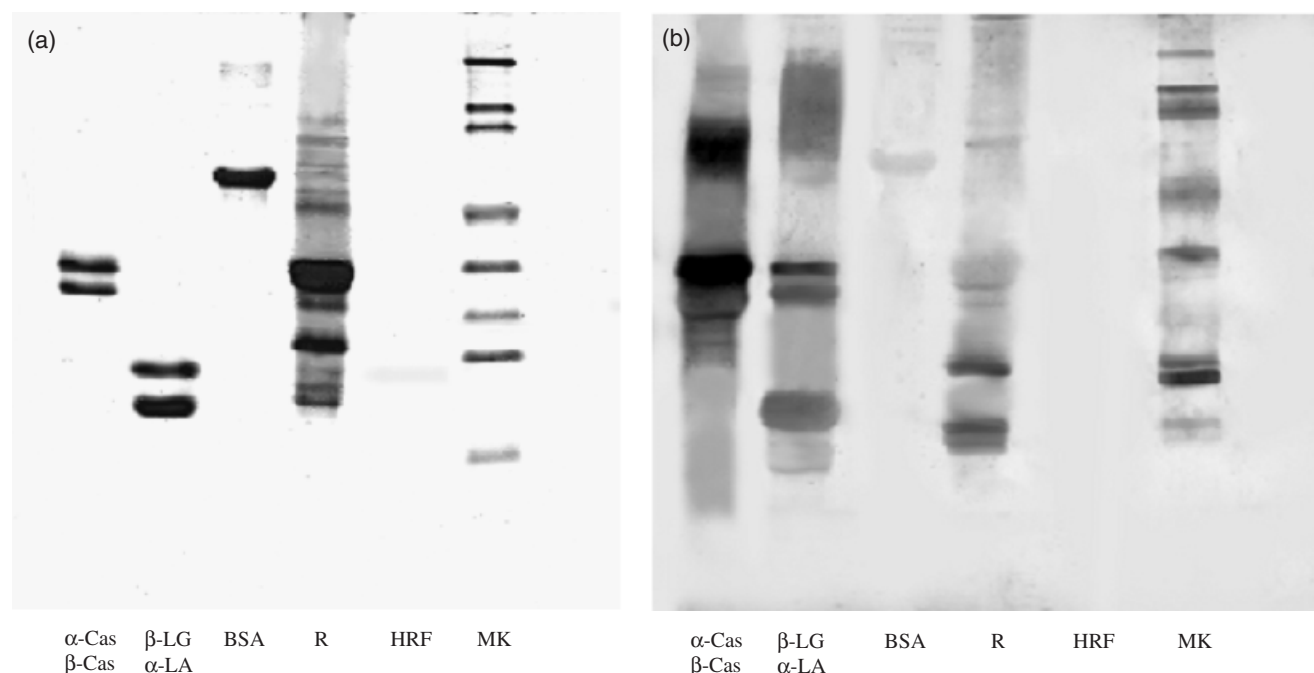


Fig. 1. Sodium dodecyl sulfate electrophoresis (a) and immunoblotting (b) obtained with purified cow's milk proteins, rice (R) and hydrolysed rice formula (HRF) from patient 24. This subject showed a significant positive reaction to rice and a negative response to HRF, both in CAP test and immunoblotting. α -LA, α -lactalbumin; β -LG, β -lactoglobulin; BSA, bovine serum albumin; MK, molecular weight marker solution.

earlier findings that children with CMA are generally sensitized to more than one milk protein and that sensitization does not depend on how much of the implicated protein fraction is present [31]. Casein traces in α -LA and β -LG may still be recognized at CAP (Fig. 1) and may elicit significant SPT weals in spite of purification for immunoblot. Proteins denatured for separation and solubility prior to electrophoresis may, as a result show reduced allergenicity when a protein contains conformational epitopes, as is the case of whey proteins. Previous data on HRF [24] reported a 44.4% rate of sensitization to rice (CAP), but the sample was limited and the children, who were all allergic to soy, were highly selected. In the present series, rice-specific IgE could be demonstrated at SPT in 4.04% of subjects sensitive to cow's milk. This suggests that a specific correlation exists between rice and soy protein sensitization. On the other hand, cross-sensitization – sometimes resulting in severe cross-reactions – has been described between cereals and legumes [32–34]. CAP and immunoblotting (but not SPT) also show a lower, although significant, level of sensitization to rice without clinical relevance for patients who could all eat rice without ill effects. That the presence of specific IgE to rice does not generally translate into symptoms appears to suggest that, as in the case of wheat, soy [35], peanut [36] and carob [37], sensitivity to these allergens is associated with poor diagnostic predictivity. Moreover, immunoblottings with HRF are negative even in children sensitized to rice, suggesting that hydrolysis modifies rice protein antigenicity inasmuch as their IgE-binding capability is abolished.

We did not set out to investigate milk substitute use, but a comment that Italian paediatricians tend to recommend soy-based products over eHF, in spite of European guidelines to the contrary [22] is inescapable. Scant use of amino acid formula is also noteworthy. However, it is worrisome (on nutritional grounds) that many children were not prescribed any cow's milk substitute, even if their disease expression was moderate. The choice of a suitable alternative to cow's milk should be assessed against the expected individual likelihood of adverse reactions. To date, the treatment of choice for CMA is soy formula or eHF [21, 22]. eHF is well tolerated by children with CMA but reactions in up to 5% of cases are not to be ruled out [21]. Any cow's milk-based formula carries a risk of residual antigenicity. Positive SPT to even extensively hydrolysed formulae have been found in children with CMA [38, 39], and there are reports of reactions to most cow's milk hydrolysates [40–44]. Moreover, eHF are often unacceptable on account of taste and cost [45]. Caution should thus be exercised before introducing any cow's milk-based hydrolysate into the diet of highly allergic children [11]. On the other hand, the use of soy formulae is limited by the possibility of soy allergy. Adverse reactions found in 10% of cases with soy and in 2.2% with eHF [15], were not found in the present series. This suggests that

HRF is not only a suitable therapeutic option for children with multiple food allergies – when the only alternative is an elemental formula – but also in the case of children with CMA, irrespective of the presence of other sensitizations. Even though the present study did not attempt a direct comparison between dietary solutions for these patients with special needs, future research should address the question of indication for each of these formulae. From an allergist's perspective, long-term trials assessing the development of possible allergic reactions to HRF against eHF and soy formula are the next step. In this context, height/weight response studies measuring their bionutritional value [46] through various age groups are necessary to confirm the nutritional adequacy of dietetic regimens across different age groups. With this further condition, the tolerance of HRF by children with CMA represents an alternative dietary opportunity, together with eHF and soy formula, even in the presence of multiple allergic sensitization.

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