
Allergenicity of a hydrolyzed rice infant formula in a guinea pig model

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Background: Because sensitization to cow's milk is a common finding in children, the identification of safe alternative protein sources is important in the management of childhood allergy.

Objective: To evaluate, in an animal model, the allergenicity of a novel formula based on hydrolyzed rice proteins.

Methods: We conducted an experiment involving 130 guinea pigs, from 7 to 12 days old at the onset of the study. The animals were divided into 13 groups and were given, ad libitum, one of the following liquids to drink: (1) rice hydrolysate formula (RF), (2) a conventional cow's milk formula (CMF), or (3) water. After a 37-day sensitization period, a challenge was given, consisting of an intravenous injection of either isolated proteins or ultracentrifuged formulas (uCMF and uRF). Specific IgG antibodies to β -lactoglobulin, casein, and whole rice protein were measured by enzyme-linked immunosorbent assay.

Results: When animals fed CMF were challenged with β -lactoglobulin, casein, or whole uCMF, they showed significantly more reactions than did those fed RF when challenged with the same proteins ($P < 0.001$). In the groups fed RF, no reaction was observed after challenge with uRF, and only 2 mild reactions occurred after challenge with rice protein. Very low levels of specific IgG antibodies to rice protein were noted in all the groups, including the RF-fed animals, and no significant differences were evident between groups.

Conclusions: The findings in this study suggest that this new formula based on hydrolyzed rice proteins has a very low sensitizing capability.

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INTRODUCTION

Sensitization to proteins in cow's milk probably is the most common food allergy in infancy, ranging in reported frequency from 0.3 to 7.5%.^{1,2} In recent years, several formulas have been proposed to prevent or to treat cow's milk allergy in children.³ Most of these formulas are produced by protein hydrolysis of cow's milk, bovine or porcine collagen, or soy. Although cow's milk hydrolysates can be effective in preventing allergic sensitization in children,^{4–7} detectable levels of serum IgE antibodies to cow's milk antigens^{8–11} and anaphylactic reactions^{12,13} have been reported, even for extensively hydrolyzed formulas.

The residual antigenic activity of "hypoallergenic" infant formulas can be investigated preclinically by in vitro evaluations, including gel-permeation chromatography, enzyme-linked immunosorbent assay or radioallergosorbent test inhibition, immunoblotting,¹⁴ and in vivo studies in animal models.^{15,16} Although results of experiments in animal models are unlikely to have a direct application to humans, such studies are required as part of the premarketing evaluation of allergenicity and immunogenicity of new hypoallergenic infant formulas.¹⁷

In this study, we evaluated the allergenicity of a novel formula based on hydrolyzed rice proteins in a guinea pig model as a preclinical investigation aimed at determining

whether, ultimately, children allergic to cow's milk will be able to consume rice hydrolysate with safety.

MATERIALS AND METHODS

In accordance with a previously validated animal model developed to test the allergenicity of infant formulas,^{16,18} 130 guinea pigs (Charles River, Milano, Italy), ranging in age from 7 to 12 days at the beginning of the experiment, were used. The animals received a regular pellet, certified free of rice and cow's milk proteins (Charles River). In addition, they received, ad libitum, one of the following liquids to drink for a 37-day period: (1) rice hydrolysate formula (RF; Plada, Milano, Italy), (2) cow's milk formula (CMF; Vivena 1, Plada), or (3) water (Table 1). The 130 guinea pigs were divided into 13 groups of 10 animals each. Supplementation consisted of CMF in 50 animals, RF in 50, and water in 30. After the 37-day period of sensitization, when the animals were 44 to 49 days old, different intravenous challenges were given for evaluation of whether the animals had developed specific sensitization to the various formulas they received as supplements. The challenge consisted of an intravenous injection of 0.5 mL of either isolated proteins casein (Sigma Chemical, St. Louis, MO) and β -lactoglobulin (Sigma Chemical), at a concentration of 6 mg/mL, or ultracentrifuged proteins. These were obtained by ultracentrifugation (10,000 g for 5 minutes) of rice proteins (obtained from Plada and containing rice proteins 71.6%, glucosides 13.1%, lipids 6.9%, minerals 0.9%, and humidity 7.5%) or from ultracentrifuged formulas (uCMF and uRF; Table 1). This experi-

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Table 1. Experimental Design and Results in 130 Guinea Pigs

Factor	Group*												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Supplement	CMF	CMF	CMF	CMF	CMF	RF	RF	RF	RF	RF	H ₂ O	H ₂ O	H ₂ O
Challenge†	uCMF	Cas	β L	uRF	RP	uRF	RP	uCMF	Cas	β L	Saline	uCMF	uRF
Reactions													
Fatal	8	10	10	0	0	0	0	0	0	0	0	0	0
Severe	2	0	0	0	0	0	0	0	0	0	0	0	0
Mild	0	0	0	0	0	0	2	0	0	0	0	0	0
None	0	0	0	10	10	10	8	10	10	10	9	9	10

Abbreviations: Cas, casein; CMF, cow's milk formula; β L, β -lactoglobulin; RF, rice hydrolysate formula; RP, rice proteins; uCMF, ultracentrifuged cow's milk formula; uRF, ultracentrifuged rice hydrolysate formula.

* Each study group consisted of 10 guinea pigs. Two animals died during sensitization period.

† The challenge consisted of a 0.5-mL intravenous injection; investigators were unaware of which substance was being injected.

mental design was intended to minimize the number of animals sacrificed in this study. For this reason, the group that had received water as a supplement was challenged only with saline (absolute negative control) and with uCMF and uRF that were obtained from the whole formulas, therefore representing the most complete spectrum of proteins contained in the supplements given to the animals to drink during the sensitization phase.

The reactions to the challenge were categorized as none, mild, severe, or fatal and labeled for analysis with a score ranging from 0 (no reaction) to 3 (fatal reaction).¹⁸ Challenges were performed in a blinded fashion to eliminate observer bias concerning nonfatal reactions. The National Institutes of Health guidelines for the care and use of laboratory animals were followed.

Specific anaphylactic reactions (IgG antibodies) to β -lactoglobulin, casein, and whole rice protein were measured by enzyme-linked immunosorbent assay.¹⁸ Briefly, 96-well microtiter plates (Nunc-Immunoplate, Intermed, Denmark) were coated with a solution of antigen in phosphate-buffered saline, incubated overnight at 4° C, and washed with phosphate-buffered saline-Tween 20. Serum samples were added at 1/10 dilution, incubated for 90 minutes at 37° C, and, after washing, re-incubated in the same conditions with 1:4000 dilution of rabbit anti-guinea pig monoclonal antibodies against total IgG, conjugated with peroxidases. Substrate was added to each well for 30 minutes, and optical density was read at 465 nm. IgG concentrations were expressed as optical density (absorbance).¹⁹

Between-group comparisons were determined with use of Student *t* test for unpaired samples. Bonferroni adjustment was applied when appropriate.

RESULTS

During the sensitization phase, the animals regularly consumed the supplementation formulas, with no evident difference between groups. In fact, the residual amounts of liquid formulas recovered in the dispensers at the time of the daily care and feeding of the animals were comparable, as were the solid pellet remaining portions.

Two animals, one in group 11 and one in group 12, died during the period of sensitization. The results of the *in vivo* challenge are summarized in Table 1. A significantly higher reactivity was observed for animals fed CMF (groups 1, 2, and 3) than for those fed RF (groups 8, 9, and 10) when challenged with β -lactoglobulin, casein, or whole uCMF ($P < 0.001$). In the groups fed RF, no reaction was noted after challenge with uRF (group 6), and only 2 mild reactions occurred after challenge with rice protein (group 7).

CMF-fed animals (groups 1, 2, and 3) challenged with β -lactoglobulin, casein, or uCMF had significantly more reactions than did animals in the water-supplemented control group challenged with uCMF (group 12; $P < 0.001$). RF-fed animals tested with any stimulus (groups 6 through 10) and CMF-fed animals challenged with uRF or rice proteins (groups 4 and 5) showed no significant difference compared with the control group challenged with uRF (group 13).

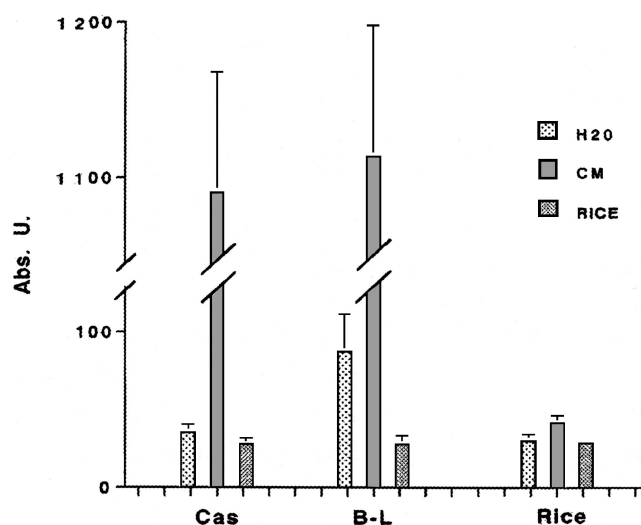


Figure 1. Levels of specific serum antibodies (IgG) to casein (Cas), β -lactoglobulin (β -L), and rice expressed as absorbance units (Abs. U.). CM, cow's milk.

Specific anaphylactic serum levels of IgG antibodies to β -lactoglobulin, casein, and whole cow's milk protein are shown in Figure 1. Animals fed CMF (groups 1 through 5) showed significantly higher levels of serum specific IgG for β -lactoglobulin and casein than did animals receiving RF (groups 6 through 10) and water (groups 11, 12, and 13; $P < 0.001$). Animals fed RF (groups 6 through 10) failed to show significantly higher levels of specific IgG antibodies to β -lactoglobulin, casein, and whole cow's milk protein than did animals receiving water (groups 11, 12, and 13). Very low levels of specific IgG antibodies to rice protein were observed in all the groups, including the RF-fed animals, with no significant difference between groups.

DISCUSSION

The management of children sensitized to cow's milk protein necessitates the exclusion of cow's milk antigens from the diet. Although soy is widely used as an alternative source of protein for infant formulas, reactions to soy may occur in 17 to 47% of children sensitized to cow's milk proteins.²⁰

Formulas based on hydrolyzed milk protein may still have residual allergenic activity, and cases of anaphylaxis have been reported in sensitized children who were consuming hypoallergenic formulas.³ The presence of serum IgE antibodies to fragments of hydrolyzed formulas recovered in allergic children⁹ could explain the occurrence of severe anaphylactic reactions in children receiving extensively hydrolyzed formulas.^{21,22} Small amounts of either residual intact proteins or peptides retaining antigenic determinants have been hypothesized to be relevant to the development of such severe reactions.²³ Therefore, the possibility of other protein sources as alternatives to cow's milk seems particularly important in the management of allergic children.

In this study, no fatal reaction occurred in either CMF- or RF-fed animals when challenged with rice proteins or the supernatant of RF. Furthermore, significantly higher levels of β -lactoglobulin and casein were observed in CMF-fed animals when compared with those given RF, but not in both RF- and CMF-fed animals as compared with water-supplemented controls.

CONCLUSION

The data from this experimental study suggest that this new infant formula based on hydrolyzed rice proteins has a very low sensitizing capability. The results of these preclinical observations cannot be directly extrapolated to humans,¹¹ but they constitute a positive indication for the extension of this investigation to a clinical phase focused on evaluation of the allergenicity of this new formula obtained from hydrolyzed rice proteins. The clinical phase studies should include double-blind, placebo-controlled challenges and they must be performed in a setting equipped for prompt treatment of any anaphylactic reactions that may occur.

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