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Determination of the allergenicity of an infant formula based on vegetal protein in the Ussing chamber

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In recent years a new preparation based on hydrolyzed rice protein has been marketed and could be a useful alternative in cases of cow's milk protein allergy. To determine the allergenicity of a hydrolyzed rice protein infant formula in the Ussing chamber, by provocative tests performed on jejunal fragments of Balb/c mice parenterally sensitized to (α -La, β -Lg). Methods: 60 Balb/c females 6-8 weeks old ($19,50 \pm 0,25$) g : 20 mice immunized with β -Lg (group 1), 20 mice immunized with α -La (group 2), 20 mice receiving no treatment (control). Samples of an infant milk based on hydrolyzed rice protein, a standard milk 1st age, cow's milk are used in this study. The sensitization is assessed by immunoenzymatic dosage (ELISA) of a specific IgG and IgE on sera obtained at day 35. The allergenicity of these samples is measured in the ussing chamber by provocative tests carried out on jejunal fragments of Balb/c mice, which are parenterally sensitized to (α -La, β -Lg). IgE and IgG anti- β -Lg and anti- α -La are produced at a significantly high level which is very strongly significant ($p < 0.001$). Our results indicate that the stimulation of the infant formula based on rice in the serous compartment of the tissues of the sensitized animals does not result any significant effect on the short-circuit current (Isc). On the other hand, the deposition of sensitizing proteins (β -Lg, α -La), cow's milk and standard infant formula significantly stimulates the Isc, indicating active chlorine secretion which suggests an anaphylactic local reaction. This preliminary results show that the hydrolyzed rice protein preparations seem to be a possible alternative and a good treatment in case of cow's milk protein allergy

Keywords: Allergenicity - Intestinal epithelium - Vegetal protein - Rice hydrolysates - Infant formulas.

INTRODUCTION

Allergy to cow's milk proteins is an exaggerated and inadequate response of the immune system to proteins that it wrongly considers dangerous. Here, it is the proteins of cow's milk that can be found in infant formula, but also in the breast milk when the mother breastfeeding consumes dairy products (Dupont, 2008).

In recent years a new useful alternative in the

case of cow's milk allergy based on extensive rice protein hydrolysates has been marketed (Fiocchi et al. 2006).

These infant formulas have been developed with a composition that complies with the European regulation of dietetic foods for medical purposes and makes it possible to cover the nutritional requirements of infants, and the taste quality of rice protein preparations is higher than that of other hydrolysates (Girardet et al. 2010).

Rice is indeed a cereal with low allergenicity and naturally devoid of phyto-estrogens (Koo and Lasekan, 2007; La Ode et al. 2018) and well suited to these indications, provided that its protein fraction is supplemented with lysine, threonine and tryptophan, in order to obtain an aminogram conforming to that of breast milk which constitutes the reference protein (Girardet et al. 2013).

Currently, the potentiality of a product to prevent allergy can not be determined, according to Espghan (European Society for Paediatric Gastroenterology, Hepatology and Nutrition) and l'Espaci (European Society for Paediatric Allergology and Clinical Immunology) just by randomized clinical studies with correct methodology, this is why this work was undertaken to study the allergenicity of a commercial infant formula based on rice hydrolysates (Modilac Expert Riz®) used in the treatment of cow's milk allergy.

MATERIALS AND METHODS

Reagents and products

The different products and reagents as well as the pure fractions of the proteins:

B-lactoglobulin (β -Lg) and α -lactalbumin (α -La) are from Prolabo, Merck and Sigma (France).

Samples used

Cow's milk

The milk used was freshly collected on a farm in the Oran region (Algeria), the raw milk (pH 6.8) collected is skimmed by centrifugation at 3500 rpm for 15 minutes at 4 ° C. This operation is intended to eliminate the fat, the skimmed milk is then freeze-dried using a lyophilizer of the Speed Vac concentrator 100H type.

Infant formulas used

Product 1

The preparation used Modilac Expert Riz® (Table 1) is a dietary food for special medical purposes in case of cow's milk protein allergy, re-feeding as a result of acute, prolonged or chronic diarrhea of malnutrition. It is a preparation based on 100% plant-free hydrolyzed rice protein without cow's milk proteins and lactose free, this product is part of the Modilac range of the Sodilac laboratory specialized in the design and manufacture of infant milks.

Product 2

Nursie® standard milk. This is an industrial infant formula or standard preparation and normal diet. This milk contains suitable cow's milk proteins (whey proteins and casein). This product is part of the Blédina range, a recognized brand in the field of infant milk in France.

Animals

The animals used in our protocols are Balb/c strain mice obtained from the Pasteur Institute of Algiers. These are congenic female mice, raised and acclimatized before any manipulation in the laboratory of Physiology of Nutrition and Food Safety in conditions of accommodation in accordance with the regulations. The experiments are carried out respecting the welfare of the animal, avoiding stress and agitation likely to interfere with the results. The experiments described in this study are in line with current Algerian animal welfare legislation.

Adjuvant

Al (OH)₃ or alum aluminum hydroxide was used as an adjuvant for its contribution to the stimulation of the Th₂ response (Petrovsky and Aguilar, 2004) to trigger a strong immune response.

Distribution of animals

60 Balb/c females are used. The mice are between 6 and 8 weeks old and weigh an average of 19.50 ± 0.25 g. The animals are divided into 3 experimental groups:

Group 1: 20 female Balb/c mice immunized with β -Lg.

Group 2: 20 female Balb/c mice immunized with α -La.

Group 3: 20 female Balb/c mice receiving no treatment. This lot constitutes the negative control group.

Immunization protocol

Group 1 and 2 mice are immunized intraperitoneally. Each mouse receives a dose of 100 μ l of a phosphate buffered saline (PBS) pH 7.4 containing 10 μ g of β -Lg or α -La depending on the group, mixed with 2 mg of Al (OH)₃. The intraperitoneal injections take place on day 0 and then, in the form of reminders and under the same conditions, at the 14th, 21st and 28th days of the protocol.

Table 1: Composition of the Modilac Expert Riz® Commercial Formula

Average analysis per 100 ml		Modilac Expert Riz®
Presentation Box		800 g
Energy value kcal		68
protein	g	1,6
Carbohydrates	g	7,6
Lactose	g	Without
Maltodextrin	g	6
Corn starches	g	1,6
lipids g		3,4
Linoleic acid	mg	444
A-linolenic acid	mg	38
Arachidonic acid	mg	No
Docosahexaenoic Acid	mg	No
Medium Chain Triglycerides	g	0,7
Calcium	mg	61
Phosphorus	mg	34
Iron	mg	0,7
Scoop	g	4,5
Reconstitution		13,5%
Osmolarity mOsmol/L		200

Blood sample

On D0 and before any manipulation of the animal, a first retro-orbital blood sample is made using a Pasteur pipette. The second sampling takes place on day 35. We harvest an average volume of 400 to 500 μ l of blood per mouse which is then centrifuged at 3500 rpm for 15 minutes at 4°C. In order to separate the serum which is then aliquoted in the Eppendorf microtubes which are stored frozen at -20°C.

Determination of serum antibodies

To assess the degree of sensitization of mice against β -Lg, α -La, serum antibodies of different immunoglobulin: IgG, and total IgE isotypes were measured. The method used is a noncompetitive method using Enzyme-Linked Immunosorbent Assay (ELISA).

Measurement of the allergenicity of the different samples

In order to evaluate the allergenicity of proteins in infant formulas, we use in vitro technique Ussing chamber. It allows us to study the interaction between the different proteins of infantile preparations with the intestinal epithelium of mice immunized with β -Lg, α -La.

Principle of the Ussing Chamber

The Ussing chamber is a fundamental method for studying and understanding the mechanisms

of intestinal transport. This experimental device was designed (Ussing and Zerahn, 1951) for the measurement of ionic fluxes through an epithelium. This protocol of measure which we adopted in our work, has been used by several authors (Saïdi et al. 1995; Brahim el al. 2012; Bouferkas et al. 2019).

Mounting fragments of mouse jejunum in Ussing Chamber

The mice are kept fasting the night before. They are anesthetized with 10% chloral, then the abdomen is opened and the entire jejunal segment is gently removed from the abdominal cavity, emptied of its contents by two or three rinses with the cold Ringer.

After having gently removed the mesentery, the jejunal segment is then incised according to the mesenteric border and then cut into fragments which are maintained in cold Ringer and oxygenated by a stream of carbogen (CO₂: 5%, O₂: 95%). Each time, a fragment is mounted between two chambers of Lucite whose opening determines the surface of the exposed intestinal mucosa (0.10 cm²). The volume of Ringer deposited in each compartment of the chamber is 5 ml, the system is maintained at 37°C. After assembly of the tissue, about 15 to 20 minutes are required to stabilize the basic electrophysiological parameters. At the end of this period, 60 μ g / ml of antigen to be tested or 60 μ g / ml powder of cow's milk or infant preparations

are deposited in the serous compartment of the chamber.

The various electrophysiological parameters are then measured initially, every minute during the first 5 minutes, then once every 5 min, during 15 min of experience.

Statistical analysis

The results are presented as mean \pm standard error ($X \pm ES$). The statistical analysis is carried out using a statistical software program called STATISTICA (5.1.2006). Analysis of the variance is carried out with the ANOVA test. The threshold of significance retained is that which is usually considered, 5%.

RESULTS

Serum titers of anti- β -Lg and anti- α -La antibodies of immunized mice

The revelation of specific IgG and IgE anti- β -Lg and α -La is carried out by an antibody-specific assay (ELISA) technique on Balb/c sera sensitized to the β -Lg and α -La ($n = 10$).

Serum IgG titers

At D0, prior to any immunization, the serum anti- β -Lg and α -La IgG serum is undetectable in the sera of the protocol mice. After 35 days of immunization, these antibodies are produced at a significantly high level, reaching a titer of 1/100,000th, which is very strongly significant ($p < 0.001$).

Serum IgE titers

The serum IgE anti- β -Lg and anti- α -La is 1/25th ($p < 0.001$). This presence of IgE in the sera of mice immunized with β -Lg and α -La translates a dependent IgE immune response.

In vitro study of the interaction of sensitizing proteins, cow's milk, standard infant formula, hydrolyzed rice infant formula with mouse mucosa sensitized to β -Lg and α -La

The aim of this study is to investigate the existence of a local anaphylactic response after stimulation of the jejunal mucosa of mice sensitized with β -Lg and α -La, with the proteins of the different infant formulas (standard, rice), cow's milk, sensitizing proteins (β -Lg and α -La).

The measurements are carried out in two steps, the first is the measurement of the electrophysiological parameters carried out in the basal state and which lasts between 10 and 15 minutes. It is done before any stimulation of the

tissues.

The second measurement is carried out after the deposition, either of the β -Lg, α -La sensitizing proteins, the cow's milk or the infant milks in the serous compartment. This measurement is performed during the first five minutes and then ten minutes after stimulation.

Effect on Isc

The Isc or short circuit current ($\mu A/cm^2$) corresponds to the algebraic sum of the ionic fluxes on either side of the intestinal epithelium. In an allergy model, its increase usually reflects an electrogenic secretion of Cl⁻ chlorine.

Effect of β -Lg and α -La on Short Circuit Isc

Jejunal fragments of β -Lg-sensitized mice are mounted in the Ussing chamber and stimulated by the deposition of the β -Lg sensitizing protein at the concentration of 60 $\mu g / ml$ in the serous compartment. There was a significant increase in Isc values after β -Lg deposition, which changed from baseline values of $40.88 \pm 5.34 \mu A/cm^2$ to $56.10 \pm 5.12 \mu A/cm^2$ ($\Delta Isc = 15.22 \pm 0.94 \mu A/cm^2$, $p < 0.001$).

For mice immunized with α -La and stimulated with α -La, base values of Isc range from $39.88 \pm 5.71 \mu A/cm^2$ to $57.004 \pm 5.34 \mu A/cm^2$ ($\Delta Isc = 17.12 \pm 0.78 \mu A/cm^2$, $p < 0.001$).

In comparison, stimulation of the intestines of naïve mice (not immunized) with β -Lg or α -La did not induce any significant variation in Isc (Table 2).

These results suggest the existence of a local anaphylactic reaction produced by the direct interaction of sensitizing antigens with the immunocompetent cells of the intestinal mucosa of β -Lg or α -La-sensitized animals.

Effect of ovalbumin on Isc

In order to show that the increase in Isc of the fragments of sensitized mice is specific to the sensitizing protein, ovalbumin was tested in the serous compartment (60 $\mu g/ml$) under the same experimental conditions. The results obtained were compared with those of the non-sensitized controls treated according to the same procedure (Fig.1).

Our results indicate that the deposition of ovalbumin in the serous compartment of tissues of sensitized animals or controls does not result any significant effect on Isc.

Effect of cow's milk on short-circuit current

The deposition of cow's milk at a

concentration of 60 mg/ml in the serous compartment stimulates the increase of *Isc* in the tissues of the β -Lg-sensitized animals, the base values are increased from $66.15 \pm 6.68 \mu\text{A}/\text{cm}^2$ at $78.95 \pm 6.74 \mu\text{A}/\text{cm}^2$ ($\Delta I_{sc} = 12.8 \pm 0.99 \text{ p} < 0.001$).

For mice immunized with α -La and stimulated by cow's milk, the *Isc* base values range from $72.89 \pm 2.96 \mu\text{A}/\text{cm}^2$ to $88.058 \pm 2.60 \mu\text{A}/\text{cm}^2$ ($\Delta I_{sc} = 15.16 \pm 0.57 \text{ p} < 0.001$).

These results suggest the existence of a local anaphylactic reaction due to the direct interaction of the sensitizing antigen (β -Lg or α -La) with the mucous membranes of the jejunal fragments.

Effect of infant formula (Standard, Hydrolyzed Rice) on *Isc*

The deposition of 60 $\mu\text{g}/\text{ml}$ of the standard milk in the serous compartment of the tissues of the animals sensitized to β -Lg causes a very significant increase of the *Isc*. The baseline values range from $53.11 \pm 6.66 \mu\text{A}/\text{cm}^2$ to $67.87 \pm 6.43 \mu\text{A}/\text{cm}^2$ ($\Delta I_{sc} = 14.76 \pm 1.66, \text{ p} < 0.001$).

The same response is obtained when the tissues of the α -La-sensitized animals are stimulated by the standard preparation, the base values increase from $24.92 \pm 0.23 \mu\text{A}/\text{cm}^2$ to $40.44 \pm 2.29 \mu\text{A}/\text{cm}^2$ ($\Delta I_{sc} = 15.52 \pm 2.06, \text{ p} < 0.001$). On the other hand, the *Isc* values remain unchanged when the tissues of the β -Lg or α -La-sensitized animals are stimulated with the hydrolyzed rice-based formula (Table 2).

Effect on conductance

Effect of β -Lg and α -La on conductance (G)

Conductance (G) values increased from $34.71 \pm 2.64 \text{ mmho}/\text{cm}^2$ to $51.21 \pm 3.53 \text{ mmho}/\text{cm}^2$ ($\text{p} < 0.001$) in mice immunized and stimulated with β -Lg and $34, 71 \pm 2.64 \text{ mmho}/\text{cm}^2$ at $51.51 \pm 3.37 \text{ mmho}/\text{cm}^2$ ($\text{p} < 0.001$) in mice immunized and stimulated with α -La.

This increase in conductance is synonymous with a decrease in resistance, by a relaxation of the mucosa. This relaxation can be explained physiologically by an increase in intestinal permeability in food protein allergy.

Effect of cow's milk on conductance (G)

We measured changes in conductance (G) after stimulation of the jejunal fragments of β -Lg sensitized mice with cow's milk. G values increased from $24.43 \pm 2.89 \text{ mmho}/\text{cm}^2$ to $31.43 \pm 2.91 \text{ mmho}/\text{cm}^2$ ($\text{p} < 0.001$). In mice immunized with α -La and stimulated with cow's milk, G values

increased from $23.33 \pm 2.97 \text{ mmho}/\text{cm}^2$ to $31.16 \pm 2.90 \text{ mmho}/\text{cm}^2$ ($\text{p} < 0.001$).

Effect of infant formula (Standard, Hydrolyzed rice) on conductance

The deposition of standard milk in the serous compartment of the tissues of the animals sensitized to β -Lg causes a very significant increase in conductance. Baseline values ranged from $19.05 \pm 1.04 \text{ mmho}/\text{cm}^2$ to $26.61 \pm 1.20 \text{ mmho}/\text{cm}^2$ ($\text{p} < 0.001$).

A very significant increase in conductance is observed after stimulation of the tissues of animals sensitized to α -La by the standard preparation. Values ranged from $16.79 \pm 1.12 \text{ mmho}/\text{cm}^2$ to $23.31 \pm 1.74 \text{ mmho}/\text{cm}^2$ ($\text{p} < 0.001$).

This increase in conductance probably reflects an alteration of the intestinal barrier in sensitized animals. On the other hand, after stimulation of animal tissues sensitized to β -Lg and α -La by the hydrolyzed rice formula, the conductance values remained stable during the experiment. For the control no significant variation of this parameter was recorded (Table 3).

Effect of furosemide

To verify the mechanisms involved in the increase of the secretory activity of the intestinal epithelium when the latter is activated by the sensitizing antigen, we tested the action of a diuretic, furosemide. This agent is a specific inhibitor of the Cl/Na/K cotransport system located on the basolateral membrane. Furosemide causes depletion of the Cl cell and thus a decrease in Cl secretion. To this end, we constructed jejunal fragments of mice immunized with β -Lg and α -La in the Ussing chamber and after stabilization of the basic electrophysiological parameters, the tissues are stimulated by furosemide on the serous side at the concentration of $5 \times 10^{-2} \text{ M}$. The tissues were subsequently stimulated with the sensitizing proteins.

The results showed that when furosemide (Fig. 2, 3) is added to the serous compartment, the *Isc* does not vary and remains comparable with the basal values. The deposition of the sensitizing antigen in the serous side does not produce any variation of the *Isc*. These results imply that the short circuit current induced by the sensitizing antigen is indeed a chlorine stream.

Effect of glucose

To test the structural integrity of the jejunal fragments used in our experiments, we tested the effect of glucose on the mucosal and serous

slopes of these tissues at the end of the concentration of 50 mM (Fig. 2, 3).
 experiments. Glucose was deposited at a

Table 2: Effect of sensitizing proteins (β -Lg, α -La) and cow's milk as well as infant milks (Standard, hydrolyzed rice) on Isc of mouse tissues sensitized to β -Lg or α -La. Study in the Ussing Chamber.

		α -La (n=10)	β -Lg (n=10)	Cow milk (n=10)	Standard milk (n=10)	Hydrolyzed Rice (n=10)
Control	T0	23,03 \pm 1,42	22,99 \pm 1,40	22,63 \pm 1,39	17,97 \pm 1,89	20,08 \pm 1,98
	T15	24,10 \pm 1,44	24,10 \pm 1,94	23,40 \pm 1,45	18,78 \pm 2,002	20,80 \pm 2,10
P		NS	NS	NS	NS	NS
Sensitized to the β -Lg	T0	40,88 \pm 5,34		66,15 \pm 6,68	53,11 \pm 6,66	38,63 \pm 4,55
	T15	56,10 \pm 5,12		78,95 \pm 6,74	67,87 \pm 6,43	39,92 \pm 4,29
P		p <0,001		p <0,001	p <0,00	NS
Sensitized to The α -La	T0	39,88 \pm 5,71		72,89 \pm 2,96	24,92 \pm 0,23	23,44 \pm 1,64
	T15	57,004 \pm 5,34		88,058 \pm 2,60	40,44 \pm 2,29	23,90 \pm 1,76
P		p <0,001		p <0,001	p <0,001	NS

The reported values are averages and their standard errors collected at time T0 (base current values) at the time of stimulation of the tissues by the sensitizing proteins (β -Lg, α -La) and by the various infant milks and after 15 min of stimulation.

NS : Not significant.
 n : Number of tissues.

Table 3: Effect of sensitizing proteins (β -Lg, α -La) and cow's milk as well as infant milks (Standard, hydrolyzed rice) on the conductance (G) of mouse tissues sensitized to β -Lg or α -La. Study in the Ussing Chamber.

		α -La (n=10)	β -Lg (n=10)	Cow milk (n=10)	Standard milk (n=10)	Hydrolyzed Rice (n=10)
Control	T0	22,09 \pm 2,01	21,89 \pm 2,07	21,89 \pm 1,91	15,16 \pm 1,48	16,40 \pm 1,65
	T15	22,42 \pm 1,90	23,02 \pm 1,75	22,92 \pm 1,95	16,39 \pm 1,32	17,25 \pm 1,51
P		NS	NS	NS	NS	NS
Sensitized to the β -Lg	T0	34,71 \pm 2,64		24,43 \pm 2,89	19,05 \pm 1,04	23,99 \pm 3,54
	T15	51,21 \pm 3,53		31,43 \pm 2,91	26,61 \pm 1,20	24,17 \pm 3,46
P		p <0,001		p <0,001	p <0,001	NS
Sensitized to The α -La	T0	34,71 \pm 2,64		23,33 \pm 2,97	16,79 \pm 1,12	13,17 \pm 0,74
	T15	51,51 \pm 3,37		31,16 \pm 2,90	23,31 \pm 1,74	13,48 \pm 0,89
P		p <0,001		p <0,001	p <0,001	NS

The reported values are averages and their standard errors collected at time T0 (base current values) at the time of stimulation of the tissues by the sensitizing proteins (β -Lg, α -La) and by the various infant milks and after 15 Min of stimulation.

NS : Not significant.
 n : Number of tissues.

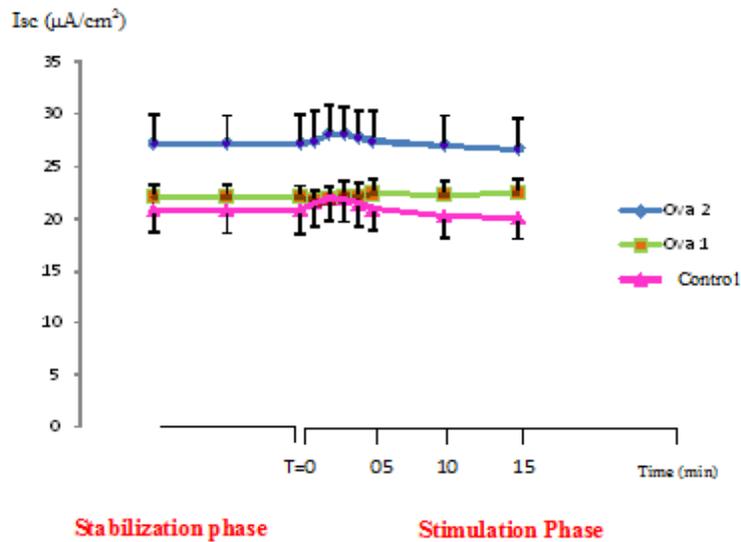


Figure 1 :Non-specific effect of ovalbumin (60µg/ml, serous side) on the evolution of the short-circuit current measured in Ussing chamber on jejunal fragments of mice immunized with β -Lg and α -La.

The values expressed are averages and their standard errors.

Ova₁ (n=10) : Animal tissues sensitized to native β -Lg and stimulated with ovalbumin.

Ova₂ (n=10) : Animal tissues sensitized to native α -La and stimulated with ovalbumin.

Controls (n=10) : Tissues of non-sensitized control animals.

* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$

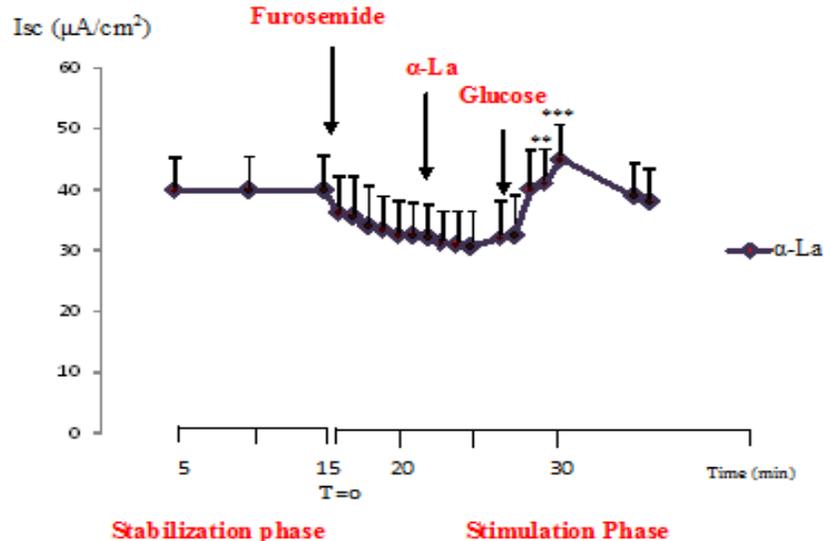


Figure 2 :Effect of α -La on the short-circuit current (Isc) measured in the Ussing chamber on jejunal fragments of mice sensitized to α -La previously incubated by the furosemide.

The values expressed are averages and their standard errors.

α -La (n=10) : Animal tissues sensitized to native α -La, stimulated by furosemide and α -La.

The glucose is added at the end of the experiment to check the integrity of the tissues

* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$

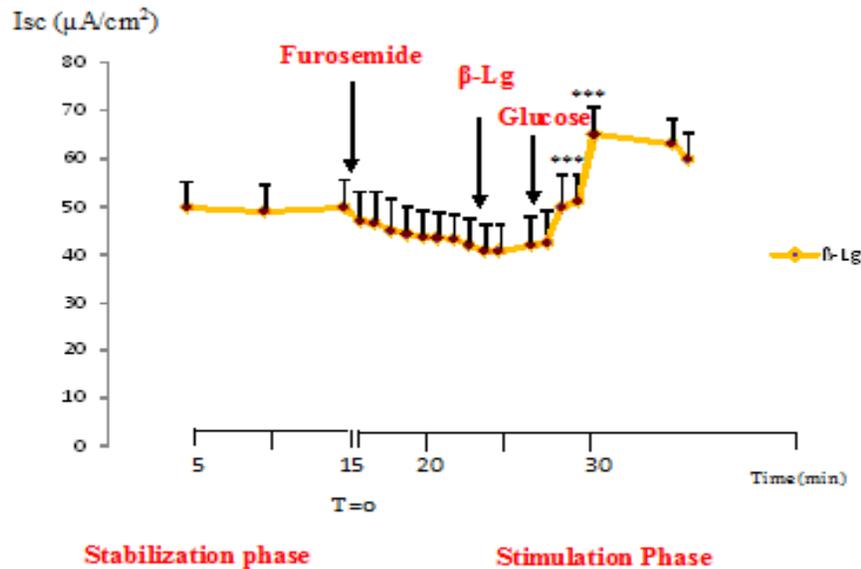


Figure 3 :Effect of the β -Lg on the short circuit current (Isc) measured in Ussing chamber on jejunal fragments of mice sensitized to the β -Lg previously incubated by the furosemide.

The values expressed are averages and their standard errors.

β -Lg (n=10) : Animal tissues sensitized to native β -Lg, stimulated by furosemide and β -Lg.

The glucose is added at the end of the experiment to check the integrity of the tissues.

* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$

The results show a very significant increase in Isc ($p < 0.001$). This indicates that the tissues are well preserved and maintain their structural and functional integrity and that furosemide does not have a toxic effect and does not interfere with the mechanism of glucose action. Therefore, the glucose- Na^+ cotransport processes and the activity of the Na^+ / K^+ ATP ase pump are not altered.

DISCUSSION

New data have recently led to changes in the management of children with cow's milk protein allergy, hydrolyzed rice protein preparations have been developed with a composition in accordance with the 2006 Directive (Directive 2006).

These preparations consist of rice protein hydrolysates enriched with lysine and threonine to obtain an amino acid profile similar to that of animal proteins. The rice protein hydrolysates complies with the European regulation of dietetic foods for medical purposes and makes it possible to cover the nutritional needs of infants, and the taste quality of rice protein preparations is

superior to that of other hydrolysates (Girardet et al. 2010).

Rice is indeed a low allergenic cereal and naturally devoid of phyto-estrogens (Koo and Lasekan, 2007). It is well adapted to these indications, provided that its protein fraction is supplemented with lysine, threonine and tryptophan, in order to obtain an aminogram conforming to that of the mother's milk which constitutes the reference protein (Directive 2006).

In recent years, these rice protein hydrolysates have been commercialized. However, no studies have confirmed their efficacy (Girardet et al. 2010). This is why this work was undertaken to study a commercial formula based on hydrolyzed rice (Modilac Expert Riz®) by determining the allergenicity of this formula in the Ussing chamber, by means of provocation tests carried out on jejunal fragments of Balb/c mice parenterally sensitized to (α -La, β -Lg). By comparing the results obtained with those obtained with the sensitizing proteins (α -La, β -Lg), cow's milk and a standard infant formula.

This work allows us to assess the degree of allergenicity of infant formulas (standard, hydrolyzed rice), in order to approximate in ousing

chamber the interaction of these infant formulas, as well as cow's milk proteins with the immunocompetent cells and to specify this action on the movements of the electrolytes (sodium, chlorine) reflected by the short-circuit current (Isc) and the conductance (G). Moreover, the electrophysiological parameters (Isc, G) make it possible to control, throughout the experiment, the viability of the tissue. Indeed, the conductance of the tissue reflects the permeability of the tight junctions with respect to the inorganic ions, mainly cations (Gumbiner, 1987). High conductance indicates tissue damage. Lastly, the addition of glucose experiment to the environment makes it easy to verify that the transport of sugars remains functional, which is another control and a proof of the viability of the tissues. Our results clearly show the development of hypersensitivity against the antigen administered β -Lg and α -La by an immune response of anti- β -Lg IgG and of high anti- α -La IgG and by a high level anti- β -Lg IgE and anti- α -La IgE found in sera from Balb/c mice. These results are consistent with those obtained by (Frossard et al. 2004) who observed in β -Lg-immunized anaphylactic mice a strong increase in serum IgG₁ and anti- β -Lg IgE titers as well as an increase in IL₄ production, reflecting a predominant immune response Th₂.

In our results, it is clear that the addition of the antigen (β -Lg or α -La) to the serous part of the jejuna of the β -Lg or α -La mice immunized in the Ussing chamber modifies the parameters electrophysiologically by significantly increasing the short circuit current.

This response is specific to the sensitizing antigen because when the jejunal fragments of immunized mice are exposed to ovalbumin, the Isc remains stable throughout the duration of the experiment. These electrical parameters are a measure of ionic movements across the epithelium and their modification also reflects the change in ion transport, mainly Na⁺ and Cl⁻.

Our results agree with those reported in the literature (Yang et al. 2000; Addou et al. 2004; El Mecherfi et al. 2015). These studies showed the significant increase in short circuit current after contacting the sensitizing antigen with the Ussing chamber mounted intestinal fragments. This increase in Isc can be translated by the fact that β -Lg, α -La, act on the tissues of the sensitized animals by increasing the electrical secretion of Na⁺ and Cl⁻, which would be one of the consequences of the passage of intact allergens or almost through the intestinal epithelium. Another study carried out on hens immunized with

bovin serum albumin (BSA) showed that in the Ussing chamber, a strong stimulation of the Isc is recorded, from the first minute of stimulation with the sensitizing protein (Caldwell et al. 2001).

In order to know the nature of the increase in Isc, the animal tissues are treated with furosemide at the concentration of 5×10^{-2} . This diuretic is known to act rapidly on the inhibition of the C1/Na/K cotransporter in the basolateral membrane of the enterocyte. No increase in Isc was observed during the deposition of furosemide, and these results make it possible to conclude that in our study model the variations of the basic short-circuit current induced by the sensitizing antigen are essentially due to a chlorine stream. This is consistent with the results of (Addou et al. 2016), the latter demonstrate that when the furosemide is added, the Isc of the jejunal fragments of mice sensitized to β -Lg and to the native whey does not vary after the antigenic stimulation. Our results also revealed a significant stimulation of the conductance in our immunized mice. This reflects damage to the intestinal epithelium at the tight junctions of the sensitized mice. Immediate hypersensitivity reactions followed by ion secretion are initiated by the interaction of antigens with IgE antibodies at the surface of the mast cell of the intestinal mucosa, releasing mediators (histamine, cytokines, etc.) which act on receptors found on the surface of epithelial cells (Rance, 2007; Merja et al. 2007). To facilitate, on the one hand, the passage of macromolecules at the tight junctions (Perdue, 1999) and increase the production of immunoglobulins specific to the milk proteins of another.

The deposition of cow's milk and standard infant milk on mouse fragments sensitized to β -Lg, α -La stimulates the short-circuit current, which translates into binding of the antigen (β -Lg, α -La) on IgE fixed on mast cells, lymphocytes, platelets and the provocation of a local anaphylactic reaction in the tissues of the sensitized mice.

Our results show that deposition of the hydrolyzed rice infant formula in the serous side of β -Lg, α -La-sensitized mice does not cause any change in the electrophysiological parameters (Isc, G), and these remain stable throughout the duration of the experiment. This confirms that the hydrolyzed rice formula does not cause cross-reactivity. This formula is a new alternative to the standard formulas based on cow's milk and soya (Fiocchi et al. 2003; Tormo et al. 2011).

According to Pramila and Rancé (2011) twelve children allergic to cow's milk proteins, the

average age of the children is nine months (extremes 4-14 months), during the consultation for an allergy test prick-tests with rice hydrolyzate Modilac rice¹® were negative in the 12 children. Similarly, in the work of Luigi et al. (2002), a hydrolyzed formula based on rice is tolerated by 18 children allergic to both cow's milk and soy protein. In the work of Girardet et al. (2010) the study of hydrolyzed rice formulas showed its good tolerance in 90% of children (mean age 4.4 months) suffering from allergy of cow's milk. The DRACMA (Diagnosis and Rationale for Action Against Cow's Milk Allergy) recommendations remain cautious about the use of hydrolyzed rice formulas as an alternative to cow's milk proteins hydrolysates in certain situations but certainly not as an alternative to synthetic amino acids. Further work is needed to better evaluate these formulas (Fiocchi et al. 2010).

CONCLUSION

In this work, we investigated the functional properties of proteins in a hydrolyzed rice infant formula (Modilac expert riz[®]) to verify if there is a cross-reactivity between extensively hydrolyzed rice proteins and antibodies directed against β -lactoglobulin and α -lactalbumin. This work allowed us to evaluate the degree of allergenicity of infant formulas (standard, hydrolyzed rice), in order to approximate in vitro the ussing chamber interaction of these infant formulas, as well as cow's milk proteins (α -La, β -Lg) with the immunocompetent cells and to specify this action on the electrolyte (sodium, chlorine) movements reflected by the short circuit current (Isc) and the conductance (G). Hydrolyzed rice protein preparations appear to be a possible alternative in case of allergy to cow's milk proteins. Further studies are needed to prove the nutritional effectiveness of these plant formulas.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

AB has set up the conception and design of the study, acquisition of data, analysis and interpretation of data, AS provided scientific

advices, DS and OK have set up drafting of the article and revising it critically for important intellectual content. All authors read and approved the final version.

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