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ORIGINAL CONTRIBUTION

Outcome of oral provocation test in egg-sensitive children receiving semi-fat hard cheese Grana Padano PDO (protected designation of origin) containing, or not, lysozyme

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Abstract
Purpose Lysozyme, obtained from egg white, is a potential food allergen used in the dairy industry to prevent late blowing of the loaf caused by the outgrowth of clostridial spores (*Cl. butyricum* and *Cl. tyrobutyricum*) during cheese aging. The aim of this study was to evaluate the possible correlation between egg protein allergy in pediatric age and sensitization to egg lysozyme, used for the preparation of Grana Padano cheese.
Methods The tolerability of Grana Padano cheese has been evaluated in pediatric patients allergic to egg proteins through an oral provocation test with increasing amounts of cheese containing, or not, lysozyme at 12 and 24 months of aging.
Results When lysozyme-sensitized children received 12-months aged and lysozyme-containing cheese, several immediate and late adverse reactions such as itching, abdominal pain, vomiting, nausea, dermatitis, rhinitis, bronchial asthma, urticaria, and angioedema were seen in 5 out of 21 subjects; only 1 out of 21 children showed an adverse reaction after challenge with 24-months-ripened lysozyme-containing cheese.
Conclusions There is a possible relationship between the severity of allergic reactions and the lysozyme-specific IgE level in blood. In particular vomiting, hypotension, and abdominal pain were present when IgE level was higher than 7 kU/L. A ripening time of 24 months may reduce allergy problems when lysozyme-containing cheese is given to sensitized subjects, probably due to the hydrolysis of antigenic epitopes during aging.

Keywords Allergy · Lysozyme · Cheese · Oral provocation test · Cheese ripening

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Introduction

After cow's milk, hen's egg allergy is one of the most common food allergies in infants and young children. Egg allergens have been described in both white and yolk, and the egg white proteins ovomucoid, ovalbumin, conalbumin, and lysozyme have been adopted in the allergen nomenclature as Gal d 1–4 [1]. The most frequent allergens reported in literature are conalbumin, followed by ovomucoid and ovalbumin. Lysozyme accounts only for 10–20 % of egg allergy [2].

In healthy subjects, the intestinal epithelial barrier plays a crucial role in the maintenance of gut homeostasis by limiting the penetration of luminal bacteria and food allergens; one of the mechanisms involved is the proteolytic activity of digestive enzymes against several protein antigens [3].

In recent years, many studies have been focused on the characterization of the structural and functional properties of lysozyme, a protein purified from egg white. Hen lysozyme is a 129 amino acid single-chain protein that hydrolyzes the

1,4 glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine of polysaccharides in the cell membrane of pathogen bacteria and saprophytes [4].

In 1992, the FAO–WHO committee approved the use of lysozyme for dairy production in the range of 10–35 g lysozyme/100 L of milk or 10–35 g lysozyme/100 kg of cheese [5].

Grana Padano is a semi-fat, hard Italian cheese recognized by the European Union as protected designation of origin (PDO). Lysozyme has been used in the production of Grana Padano cheese in order to prevent late blowing caused by the outgrowth of clostridial spores (*Cl. butyricum* and *Cl. tyrobutyricum*) present in raw milk [6–8]. Lysozyme has been recently classified as a food allergen, and the enforcement of more restrictive legislations on food allergy prompted both the dairy industry to increase the production of lysozyme-free cheese and the governments to support researches on the importance of food allergens [9]. Iaconelli et al. [10] recently studied the immunologic response of adult subjects, allergic to egg proteins, to the ingestion of Grana Padano cheese ripened for 18 months. The authors did not report any adverse reactions, while higher lysozyme levels in the blood of egg-sensitive subjects were observed. In contrast to these findings, Fremont et al. [11] previously showed an increasing specific antilysozyme IgE production in the serum of egg-sensitive pediatric patients challenged with raw egg white and pure lysozyme. Only one patient had previously facial and laryngeal angioedema after the ingestion of grated Gruyere, containing lysozyme. The authors recommended that the presence of lysozyme should be indicated on every dairy product label [10].

The prevalence of food allergy is higher in children than in adults [12, 13], and the severity of symptoms is higher in pediatric subjects. Hen's egg is a versatile ingredient used in food from many cultures, including a wide range of manufactured food products. The dietary avoidance of egg can thus be challenging for parents but it is also a relevant issue for meal production in schools or kindergartens canteens. For this reason, our study aimed to evaluate the possible correlation between egg protein allergy in children and sensitization to egg lysozyme (presence of lysozyme-specific IgE), used for the preparation of Grana Padano cheese. The effect of different cheese aging (12 or 24 months) on lysozyme sensitization was studied as well.

Materials and methods

Cheese

Grana Padano and Trentin Grana are traditional Italian cheeses, manufactured according to the same productive technique, under the control of the Grana Padano Consortium. The only difference between them is the use of lysozyme that is not allowed in the production of Trentin Grana. Grana Padano Consortium provided us with two different types of Grana Padano cheese: standard Grana Padano (manufactured with lysozyme, provided by Fordras, Lugano, CH) and Trentin Grana (lysozyme-free cheese). For both cheese, samples with two different aging periods (12 and 24 months) were provided. The amount of lysozyme in 12- and 24-month-old cheese was of 121.1 and 130.3 mg/kg, respectively. Cheeses were produced from the same milk batch, and the chemical composition of cheeses is reported in Table 1.

Patients enrollment

For the present study, 54 pediatric patients (22 girls and 32 boys), previously followed by the Paediatric Unit of the University Hospital of Pavia, Italy, were enrolled. Their age was between 2 and 13 years (mean age, 7.5 years). All patients were allergic to egg proteins and, in the previous year, they had all undergone an oral provocation test to egg that was positive (University of Pavia, number 2–2009).

All patients underwent skin prick tests for the most common food and inhalant allergens and for lysozyme as well. The skin prick test for lysozyme was negative in all patients, while 21 patients allergic to egg proteins (mean age, 6 years; range, 2–12 years) had specific antilysozyme IgE (range, 0.43–42.6 kU/L); the remaining 33 patients (mean age, 8 years; range, 3–13 years) showed no serum sensitization to lysozyme. Specific serum IgE was evaluated as described by Ferrari et al. [14]. A specific IgE level greater than 0.35 kU/L was considered positive. As previously recommended by Sampson, we considered the cutoff value of 7 kU/L for total egg-specific IgE to predict true allergy to eggs, in sensitized patients [15].

The challenge was part of the unit's clinical activity and had the aim to verify the possibility for these children to occasionally consume lysozyme-containing foods.

The research was carried out in double blind, and each parent of the children involved in the test received clear explanations and signed a written informed consent; the University of Pavia Ethical Committee approved the research.

Double-blind oral challenge with Grana Padano and Trentin Grana cheese

All patients underwent oral provocation test at least 48 h apart from each other with both cheeses.

All patients enrolled in the study consumed increasing the doses of cheese, every 20 min, according to the following scheme: 0.5 g; 1 g; 2 g; 4 g; 8 g; 14.5 g. The total

Table 1 Chemical composition of the tested cheeses (g/100 g of product)

Cheese	Aging (months)	Moisture	Protein	Fat	Ash
GP, with lysozyme	12	33.60	31.91	28.51	4.40
TN, without lysozyme	12	34.23	31.50	28.04	4.37
GP, with lysozyme	24	31.00	32.90	30.01	4.54
TN, without lysozyme	24	33.37	32.22	28.53	4.11

amount of ingested cheese was 30 g in 1 h 40 min, when lysozyme-containing cheese was eaten, the estimated intake of lysozyme was of 3.63 and 3.90 mg.

The oral provocation test was stopped before the highest dose was reached, if objective clinical symptoms, such as urticaria, angioedema, wheezing, rhinitis or conjunctivitis, vomiting, and/or diarrhea, were observed.

After the test, patients remained in the hospital for at least 2 h to evaluate the appearance of possible immediate adverse reactions, such as oral itching, abdominal pain, vomiting, nausea, dermatitis, rhinitis, bronchial asthma, urticaria, angioedema.

Experienced clinicians explained to the parents how to recognize possible late adverse reactions such as dermatitis, itching, abdominal pain, gastrointestinal symptoms. Late adverse reactions were retrospectively evaluated with a follow-up examination 48 h after the test.

Statistical analysis

Statistical analysis was performed using the SAS 9.2 software (SAS Institute, Cary, NC, USA). The difference between means was analyzed with the χ^2 test. The correlation between specific IgE was carried out using PROC REG.

Results

When challenged with lysozyme-containing cheese (12 months), children not producing lysozyme-specific IgE showed adverse reactions such as dermatitis (at least an increase of 10 points in the SCORAD score), itching, oral symptoms with spontaneous remission or remission after specific therapy; similar local reactions occurred in 2 children challenged with Trentin Grana (lysozyme-free cheese). However, no significant adverse effect could be attributed to the presence of lysozyme in the cheese (Table 2).

On the other hand, children with lysozyme-specific IgE (nine children) experienced rush or nausea, vomiting, and abdominal pain when challenged with Grana Padano cheese (Table 3). In particular, one patient after the provocation test with Grana Padano cheese containing lysozyme reported a serious anaphylactic reaction (hypotension, vomiting, abdominal pain) and was treated with intramuscular adrenaline.

No adverse symptom was observed in patients undergoing a provocation test with lysozyme-free cheese (Table 3).

In lysozyme-specific IgE negative subjects, no adverse reaction was observed when the oral provocation test was performed with the 24-months-ripened cheese, which could be due to the degradation of lysozyme epitopes during aging. However, one patient with positive lysozyme-specific IgE had a severe reaction when challenged with the 24-month-old lysozyme-containing cheese (Table 4).

Table 5 summarizes in detail all the adverse reactions observed during the provocation test. There is a relationship between the severity of allergic reactions and the levels of serum-specific IgE to lysozyme. Vomit, hypotension, and abdominal pain were present when IgE levels were higher than 7 kU/L.

The total level of IgE did not correlate with the concentration of specific IgE for ovalbumin, lysozyme, or whole yolk (Table 6).

Specific IgE levels for lysozyme did not correlate with specific IgE for albumin ($r = 0.114$), yolk ($r = 0.108$), and total IgE, while a strong relationship between specific IgE for ovalbumin and yolk was shown from our results (Table 6).

Histamine and other biogenic amines may play a role in the development of moderate cutaneous rushes. Table 7 reports the content of these molecules in the administered cheeses, and we could attribute the 2 transitory cases of urticaria observed in children receiving lysozyme-free cheese to the high content histamine of the product (171 mg/kg). Furthermore, the most aged sample of Grana Padano, containing 134.51 mg/kg of histamine could explain the 2 cases of urticaria observed in children challenged with Grana Padano [16, 17]. Nevertheless, these 2 adverse reactions could also be due to the presence of lysozyme.

Discussion

The prevalence of lysozyme sensitivity in white egg-sensitized subjects is quite variable, ranging from 22.5 to 33 % [18, 19]; Fremont et al. [11] sampled 52 people aged from 6 months to 45 years, and 19 of them (36.54 %) showed positive-specific lysozyme IgE. The prevalence observed in our study (21 out of 54 patients, 38.89 %) is similar to the

Table 2 Results of the oral provocation tests, with the 12-month-old cheese, in children without specific IgE to lysozyme

Type of cheese	Adverse symptoms (dermatitis, itching, labial symptoms)[a]	No symptoms	*P* value for χ^2
GP, with lysozyme	3	30	NS
TN, without lysozyme	2	31	

[a] Spontaneous remission or remission after local therapy

Table 3 Results of the oral provocation tests in children with positive-specific IgE to lysozyme, challenged with the 12-month-old cheese

Type of cheese	Severe adverse symptoms[a]	Mild adverse symptyoms[b]	No symptoms
GP, with lysozyme	6	3	12
TN, without lysozyme	0	0	21
P value of χ^2	0.01	0.05	–

[a] Remission after oral or intravenous antihistaminic and steroid therapy

[b] Spontaneous remission

Table 4 Results of the oral provocation tests in children with positive-specific IgE to lysozyme, challenged with the 24-month-old cheese

Type of cheese	Severe adverse symptoms[a]	Mild adverse symptoms[b]	No symptoms
GP, with lysozyme	1	2	18
TN, without lysozyme	0	0	21
P value of χ^2	NS	NS	–

[a] Remission after oral or intravenous antistaminic and steroid therapy

[b] Spontaneous remission

results shown by Fremont et al. [11], but it is almost twice the findings published by Walsh et al. [19].

The percentage of lysozyme-sensitized children showing severe adverse reactions after the oral provocation test with the 12-month-old cheese is almost 28 % (6 out of 21) while 3 subjects (14.3 %) reported moderate or self-limited mild reactions (oral itching and lip erythema). This is an important finding, since it suggests that the presence of lysozyme-specific IgE is a required but not sufficient condition for the development of severe reactions to lysozyme-containing cheese.

With the exception of one patient, all the other children with a mild sensitization to lysozyme (lysozyme-specific IgE levels between 0.36 and 5.54 kU/L) well tolerated Grana Padano cheese containing lysozyme.

We highlighted a cutoff value of 7 kU/L for lysozyme-specific IgE, above which patients may experience an increased risk of developing severe allergic reactions, and such a finding confirms the diagnostic decision points recommended by Sampson [15].

However, patients with positive lysozyme-specific IgE (5.5 kU/L) tolerated both cheeses, with and without lysozyme, and a patient with positive lysozyme-specific IgE (2.16 kU/L) showed a mild adverse reaction characterized by as urticaria, itching, and abdominal pain, only after the test with cheese containing lysozyme.

The cutoff value of specific IgE, able to predict severe adverse reactions, as observed in our study, is concordant with other data from Sampson and Ho [20] and Celik-Bilgili et al. [21] who reported a predictive value of specific IgE for egg allergy of 6 and 12.6 kU/L, respectively. Fremont et al. [11] reported positive provocation tests in patients with lysozyme-specific IgE of 13.4 kU/L but not with levels of 9.6 kU/L or less. On the other hand, Boyano Martinez et al. [22] proposed a very low threshold: 0.36 kU/L.

Our patients showed a better tolerability with the 24-month-old Grana Padano than with the 12-month-old cheese. This result could be related to the progressive degradation of antigenic epitopes of lysozyme [23] or with the formation of lysozyme conformational isomers during aging [24]. Only one patient with high levels of lysozyme-specific IgE (42.6 kU/L) developed a mild allergic reaction (abdominal pain and vomiting) after oral challenge with the 24-month-old Grana Padano. His serum IgE level was higher than the IgE levels of all the patients who developed a severe reaction.

It is so far impossible to find a strict correlation between the severity of allergic reactions and serum lysozyme-specific IgE levels, but we can speculate that the association of low lysozyme-specific IgE levels and an advanced aging of the cheese may be related to a reduced the risk of mild and severe allergic reactions in pediatric patients allergic to egg proteins and sensitized to lysozyme.

The absence of correlation between the levels of lysozyme- and ovalbumin-specific IgE is in disagreement with the findings of Walsh et al. [19], which reported a link in the response to ovalbumin and lysozyme in egg-sensitized children. In the same way, lysozyme-specific IgE did not correlate with total IgE, while Fremont et al. [11] reported a significant relationship between these two parameters. In addition, the link between ovalbumin- and egg yolk-specific IgE that we observed was not reported by Fremont et al. [11] or Walsh et al. [19].

The lysozyme molecule hosts different Ig recognition sites [22, 24–27] that can be recognized by different immunoglobulins, with different possibilities of cross-

Table 5 Symptoms occurring during the provocation test, in patients showing severe adverse reactions

	Cheese dose (g)	GP with lysozyme 12 month aged	Lysozyme-specific IgE
Patient 1	0.5	–	10.4 kU/L
	1	–	
	2	–	
	4	–	
	8	Nausea, vomiting, abdominal pain	
	14.5	Test interrupted	
Patient 2	0.5	–	2.16 kU/L
	1	–	
	2	–	
	4	–	
	8	–	
	14.5	Urticaria, itching	
Patient 3	0.5	–	7.4 kU/L
	1	–	
	2	–	
	4	Anaphylaxis: hypotension, nausea, abdominal pain	
	8	Test interrupted	
	14.5		
Patient 4	0.5	–	10.3 kU/L
	1	–	
	2	–	
	4	–	
	8	–	
	14.5	Laryngeal angioedema without urticaria	
Patient 5	0.5	–	7.8 kU/L
	1	Nausea, abdominal pain, itching	
	2	Test interrupted	
	4		
	8		
	14.5		
Patient 6	0.5	Vomiting, nausea, abdominal pain	42.6 KU/L
	1	Test interrupted	
	2		
	4		
	8		
	14.5		
		GP with lysozyme 24-month old	IgE
Patient 6	0.5	–	42.6 kU/L
	1	–	
	2	–	
	4	Nausea, vomiting, abdominal pain	
	8	Test interrupted	
	14.5		

reaction with other proteins. Furthermore, the techniques used and the purity of antibodies can differ between studies, and this could have determined the observed differences. In addition, the cited authors performed their experiment using pure lysozyme, while, in our clinical trial, patients received lysozyme embedded in a food matrix, and after at least 12 months of aging, such a difference should be considered as well.

Table 6 Correlation (*r*) between IgE-specific levels of ovalbumin (IgE-alb), lysozyme (IgE-lyso), egg yolk (IgE-yolk), and relative sum (Total-IgE)

	Total-IgE	IgE-alb	IgE-lyso	IgE-yolk
Total-IgE		−0.097	0.261	0.079
IgE-alb			0.114	0.846**
IgE-lyso				0.108
IgE-yolk				

** $P < 0.01$

Table 7 Biogenic amines content (mg/kg of product) of the administered cheese

Amines	Without lysozyme		With lysozyme	
	12 months	24 months	12 months	24 months
Putrescine	4.57	5.27	6.88	5.80
Cadaverine	0.60	1.82	3.69	4.14
Histamine	171.00	10.45	44.43	134.51
Tiramine	7.99	1.11	5.70	3.69
Spermidine	0.57	0.37	0.51	0.43
Spermine	5.22	6.75	3.80	4.19
Total	189.95	25.77	65.01	152.76

Conclusions

Egg-sensitized children, not presenting lysozyme-specific IgE, may eat lysozyme-containing cheese without the risk of adverse reactions.

The occurrence of severe adverse reactions, after the ingestion of lysozyme-containing cheese (Grana Padano), is not related to the presence of lysozyme-specific IgE, but a titer higher than 7.0 kU/L is required to have an allergic response.

Grana Padano cheese ripened more than 24 months may give fewer problems than less ripened cheese. Protein hydrolysis during the long period of ripening could break down the epitopes responsible for the occurrence of adverse reactions.

Biogenic amines can be responsible for certain forms of pseudo-allergy, and their role cannot be neglected.

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