

Oral tolerance elicited in mice by β -lactoglobulin entrapped in biodegradable microspheres

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Abstract

Oral administration of antigen is known to be appropriate for some vaccine purposes as well as oral tolerance induction. In the present study, oral administration of β -lactoglobulin (BLG) loaded poly(D,L-lactide-co-glycolide) (D,L-PLG) microspheres induced tolerance was evaluated. A single feeding of 5 μ g of encapsulated BLG tolerised BALB/c mice to subsequent BLG parenteral challenge, suppressing the specific humoral, intestinal and cellular responses. The tolerogenic efficient dose was then reduced 10,000 times, compared to oral administration of soluble BLG. This suggests that loading food proteins into D,L-PLG microspheres might be a potential tool for inducing oral tolerance with allergens. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Oral tolerance; β -Lactoglobulin; Microspheres

1. Introduction

Biodegradable microspheres built with poly(lactide-co-glycolide) (D,L-PLG) polymer has allowed encapsulation of various antigens and it has been well demonstrated that the use of these controlled release antigen delivery systems allows progress within the oral vaccine field [1–5]. Indeed, antigen vectorisation provides advantages such as protection from gastric acidity and gut proteolysis, targeting of M-cells within Peyer's Patches and enhancement of antigen phagocytosis [6,7]. These advantages, first promoted for vaccine development, now appear attractive to improve the oral tolerance immune response, which has similar needs [8,9].

Oral administration of either self-antigens or food allergens has been demonstrated to provoke specific

hyporesponsiveness of the immune system and is thus called oral tolerance. For the last decade, this strategy has been proposed as a promising treatment of autoimmune disorders and food allergies. To date, oral tolerance protocols in humans have mainly focused on autoimmune diseases, such as multiple sclerosis [10]. Regarding food allergy, cow's milk allergy is a major issue when considering the high incidence of this allergy in the pediatric newborn population (0.5–10%) [11]. Several approaches are currently under evaluation to overcome this pathology and, among these, induction of oral tolerance to milk proteins in infants, at risk of atopy, presents an interesting option for the prevention of this food allergy. Using oral administration of β -lactoglobulin, one of the major allergens in cow's milk, we have been able to demonstrate a capacity to induce specific oral tolerance in a mice. In this experimental model, the conditions of oral tolerance induction to soluble β -lactoglobulin, in particular feeding dose concentration and challenge adjuvant were optimised in order to get determinant IgE

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response in serum and intestinal secretions of the control mice [12]. The tolerogenic response was found to be dose dependent as previously observed for other antigens, such as ovalbumin [13]. In addition, the optimal age of the mice for oral tolerance induction was determined as being three weeks old, corresponding to the mouse weaning time.

Having already well defined our experimental model, we were tempted to use recent advances in oral vaccine technology to improve the efficiency of oral tolerance. The present study showed that encapsulation of β -lactoglobulin into D,L-PLG microspheres also achieved the induction of specific oral tolerance in BALB/c mice, providing a substantial decrease of the inductive dose.

2. Materials and methods

2.1. Animals

Female BALB/c mice were obtained from IFFA-Cr  do (L'Abresle, France) and experiments commenced at 3 weeks of age. The mice were bred and raised on a cow's milk free diet.

2.2. Antigens

BLG (3 $\times$ crystallised) and Ovalbumin (OVA) (Grade V) were obtained from Sigma Chemical Co.

2.3. Preparation of BLG loaded microspheres

Microspheres of D,L-lactide-co-glycolide (D,L-PLG) (ratio 75/25, MW: 19,500, Birmingham Polymers, Birmingham, AL) were prepared using the double emulsion (w/o/w) solvent evaporation technique as previously described [14]. Briefly, 20 mg of BLG was dissolved in 0.4 ml of TRIS buffer (20 mM, pH 7.5) (W1). The solution was emulsified with 2.5 ml of methylene chloride solution (O) containing 250 mg of PLG using an Ultraturrax at 13,500 rpm for 2 min to form a W1/O emulsion. This primary emulsion (inner emulsion) was poured at once into an 80 ml beaker containing 20 ml of a PVA (Poly(vinyl alcohol)(Mowiol)) (Aldrich Chemical, France) solution (2%, w/v) (external aqueous phase) (w2) and homogenised using an Ultraturrax (13 500 rpm for 2 min). This resulting W1/O/W2 emulsion was stirred at 1000 rpm for three hours at room temperature and atmospheric pressure to allow solvent evaporation and microparticle formation.

Microspheres were loaded with BLG, or they remained unloaded for control purposes. The microspheres were isolated by centrifugation (4000 rpm for 10 min), washed three times with double-distilled water

Table 1

Oral tolerance induction procedures in 6 groups of 10 BALB/c mice each

Feeding	Group	BLG (amount/mouse)	Microspheres
Saline	A	0	0
Encapsulated BLG	B 1	5 mg	yes
	B 2	500 μ g	yes
	B 3	50 μ g	yes
	B 4	5 μ g	yes
	B 5	0.5 μ g	yes
Soluble BLG	C 1	50 mg	no
	C 2	5 μ g	no
Blank microspheres	D	0	yes

and freeze-dried. The final product was stored at 4°C. Size distribution of fresh and freeze-dried particles redispersed in double-distilled water was determined using a Coulter Multisizer II. The mean size of unloaded particles was 7.8 μ m in diameter. The mean size of loaded microspheres particle, 7.2 μ m in diameter, was optimal for oral delivery [1].

The level of BLG encapsulated was determined as previously described [15]. Briefly, weighed amounts of freeze-dried microparticles were dispersed in 0.1 M NaOH containing 1% (w/v) SDS, to give a concentration of 5.0 mg per 1 ml. The suspension was shaken at room temperature for 24 h. Samples were centrifuged (4000 rpm for 10 min) and the BLG concentration was determined in the supernatant by a Bio-Rad DC protein microassay against a series of BLG standards prepared in 1 ml 0.1 N NaOH, SDS 1%. Protein assay sensitivity was 10 μ g/ml. The encapsulation efficiency was expressed by the ratio between the actual and the theoretical loading (actual BLG loading/theoretical BLG loading $\times$ 100). All samples and standards were assayed in triplicate. The actual loading was 3.8 mg/100 mg of polymer. For further characteristics of these BLG loaded microspheres see L  o et al. [15].

2.4. Induction of oral tolerance and immunisation procedures

On day 1, mice were orally administered, by gastric feeding, saline water (group A), various amounts of D,L-PLG microspheres loaded with BLG (3.8 mg BLG /100 mg of polymer) (group B), soluble BLG (group C), or unloaded microspheres (group D), as detailed in Table 1. Gavage products were suspended in 300 μ l of saline water. All mice were immunised on day 5 by intra-peritoneal administration of 0.08 mg of BLG and 0.08 mg of OVA, in 0.04 ml sterile saline mixed with 0.16 ml 2% Al(OH)₃ (Superfos Biosecotr A/S, Denmark). On day 27, a delayed type hypersensitivity (DTH) evaluation was done by duplicate thickness

measurements of the left, rear footpad prior to and following, intradermal immunisation with 0.1 mg of BLG diluted in 0.05 ml of sterile saline, using a dial gauge microcaliper (Mitutoyo, MFG, Japan).

2.5. Evaluation of the immune response

At day 28, the immune responses were evaluated in the different groups, as previously described [12]. Briefly, individual DTH responses were measured and thickness was expressed in millimetres. Then, blood samples were obtained by aortic puncture. Immediately after death, intestinal contents were collected, according to the method described by Elson [16]. Briefly, intestines were individually removed from the end of the duodenum to the end of the ileum and immediately placed in 5 ml chilled PBS-1% gelatine. They were longitudinally opened and surfaces were carefully scraped after addition of 3 ml of 0.1 mg/ml trypsin inhibitor (Sigma) in 50 mM EDTA. A first centrifugation was done on the collected secretions, 10 min at $650 \times g$, to remove major remains. 6 ml of supernatants, added to 0.03 ml of phenyl-methyl-sulfonyl-fluoride (PMSF) diluted in 95% ethanol, were then ultracentrifuged for 20 min at $27,000g$, $4^\circ C$, to clarify the secretions. Another 0.02 ml of the PMSF solution and 0.02 ml of 1% Na azide were added to each 2 ml of the clarified solutions. 15 min later, 0.2 ml of foetal calf serum (FCS) were added to the sample. Blood and intestinal samples were rapidly frozen at $-20^\circ C$ until assays. Spleens were removed and pooled according to group of treatment in 20 ml chilled RPMI completed with 5% FCS. Specific and non specific splenocyte proliferation assays were performed for each group.

2.6. Specific IgE antibody assays

Serum (three fold dilutions) and intestinal (two fold dilutions) samples were assayed in duplicate for both anti-BLG and anti-OVA IgE antibodies by ELISA as described [12]. Briefly, Nunc Maxisorp F96 microtitre plates were coated with either BLG (0.5 mg/ml) or OVA (5 mg/ml) diluted in carbonate-bicarbonate buffer, pH 9.6. Serial dilutions of samples were assayed in duplicate. Specific IgE was detected using a rat anti-mouse IgE peroxidase labelled antibody (Harlan Sera-Lab, USA). Pooled samples, either serum or intestinal contents, from twenty non-immunised mice were used as negative controls on each plate. The cut-off dilutions were determined by calculating the dilution of the samples which gave twice the absorbance of the negative controls. Titres were expressed as the $\log_{10}$ of the reciprocal of the cut-off dilution.

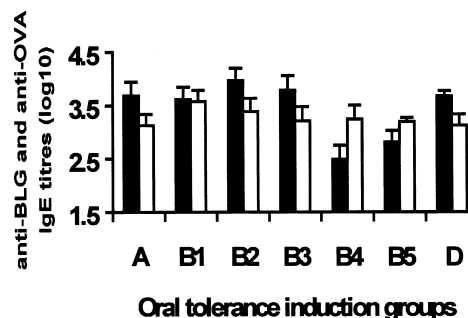


Fig. 1. Anti-BLG (black bars) and anti-OVA (white bars) IgE antibody titres in mice fed saline (A), encapsulated BLG: B1 (500 μg BLG/g of body weight), B2 (50 μg BLG/g of body weight), B3 (5 μg BLG/g of body weight), B4 (0.5 μg BLG/g of body weight), and B5 (0.05 μg BLG/g of body weight) or blank microspheres (D). Results are geometric means $\pm$ S.D. from 10 mice per group.

2.7. Cell cultures

Splenocyte proliferation assays were performed, as described [12]. Spleen cell solutions were homogenised through a cellular sieve and purified from red cells using Histopaque density centrifugation (Sigma). In 96-well microtitre plates (Costar), cells were cocultured for 120 h (5×10^6 cells/ml) in complete RPMI-10% FCS media, in the presence of BLG (Sigma) (5 to 0.1 mg/ml) or phytohaemagglutinin A (PHA) (Seromed) (250 to 2 μg /ml). Proliferation was determined by adding ^3H -thymidine [^3H]Tdr during the final 6 hours of culture. Plates were harvested and analysed by scintillation counting (TopCount, Canberra Packard, Zurich). Stimulation indices were calculated as the ratio of blank-subtracted test and control values expressed as the mean cpm (^3H)Tdr incorporation by triplicate cultures.

2.8. Statistics

DTH responses, serum and intestinal IgE responses were compared using one-way ANOVA (single factor) tests.

3. Results

As shown in Fig. 1, oral feeding of encapsulated BLG induced serum anti-BLG IgE suppression. Significant oral tolerance to BLG was reached when the oral dose of BLG loaded into the microspheres was lower than 5 μg /g of body weight. Specifically, the optimal oral dose was determined to be 0.5 μg loaded BLG/g of body weight (B4), resulting in lower anti-BLG IgE at 2.49 ± 0.27 when compared to 3.69 ± 0.25 in mice fed only saline ($p < 0.05$). Oral administration of a lower dose (B5) did also suppress specific anti-BLG IgE, albeit less pronounced.

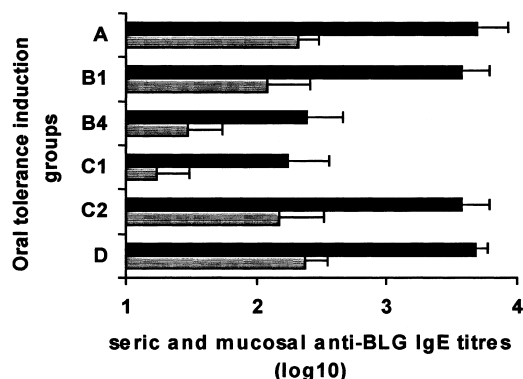


Fig. 2. Humoral (black bars) and mucosal (grey bars) anti-BLG IgE titres in mice fed saline (A), encapsulated BLG (B1 (500 μ g BLG/g of body weight) and B4 (0.5 μ g BLG/g of body weight)), soluble BLG (C1 (5 mg BLG/g of body weight) and C2 (0.5 μ g BLG/g of body weight)) or blank microspheres (D). Results are geometric means $\pm$ S.D. from 10 mice per group.

Regarding the specificity of the oral tolerance phenomenon, this experiment showed that, when compared to the control saline fed group (A), oral administration of unloaded microspheres (D) had no effect on the anti-BLG IgE responses. Conversely, titres of serum IgE against OVA, tested as a non-related antigen, remained unchanged after any of the feedings of BLG loaded microspheres.

Results reported in Fig. 2 compare oral tolerance induction due to feeding either encapsulated or soluble BLG and show that feeding of 0.5 μ g BLG encapsulated in PLG microspheres/g of body weight (B4) or soluble BLG at the optimal dose (5 mg of soluble BLG/g of body weight (C1)) were as efficient in the induction of oral tolerance. Both lead to a significant decrease of specific anti-BLG IgE in serum compared to controls (A) ($p < 0.05$). Encapsulation of BLG in D,L-PLG microspheres then allowed the reduction of the tolerogenic oral dose of BLG. When encapsulated, the optimum dose was found to be 10 000 times less than required for a soluble BLG gavage. Indeed, no significant difference was observed between data from groups B4 and C1 ($p > 0.05$). The specific decrease of anti-BLG IgE observed in group B4 could not be reached by the same amount of soluble BLG (C2). It should be noted that the highest amount of encapsulated BLG that could be fed to a mouse, 0.5 mg/g of body weight (B1), was not able to induce suppression.

Figure 2 also shows that both oral administration of optimal doses of encapsulated BLG (B4) and soluble BLG (C1) induced a significant decrease in the level of intestinal anti-BLG IgE (1.48 ± 0.26 (B4) and 1.24 ± 0.25 (C1) versus 2.32 ± 0.16 (A) ($p < 0.05$)). In our model, the magnitude of suppression of the intestinal IgE response was comparable to that observed at the humoral level. Just as for serum IgE, oral feeding of inappropriate doses of encapsulated or soluble BLG

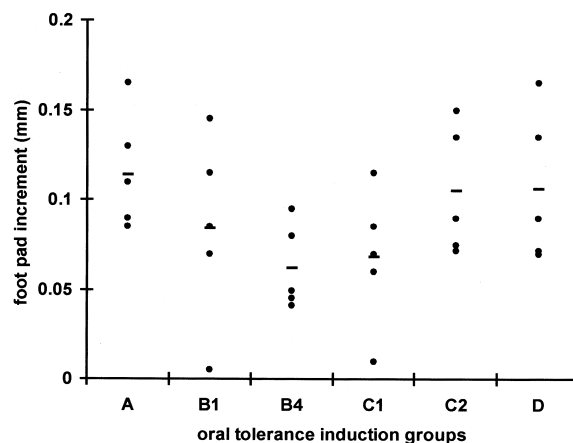


Fig. 3. Induction of BLG-specific DTH oral tolerance by single oral administration in BALB/c mice with saline (A), encapsulated BLG (B1 (500 μ g BLG/g of body weight) and B4 (0.5 μ g BLG/g of body weight)), soluble BLG (C1 (5 mg BLG/g of body weight) and C2 (0.5 μ g BLG/g of body weight)), or blank microspheres (D). (●) are individual foot pad increments and (—) are means from 5 mice per group.

did not allow modulation of the specific intestinal IgE response (B1 and C2). Finally, oral administration of blank microspheres had no effect on intestinal specific IgE levels (group D).

DTH cell-mediated responses are presented in Fig. 3. In mice fed either 5 μ g BLG encapsulated into bio-

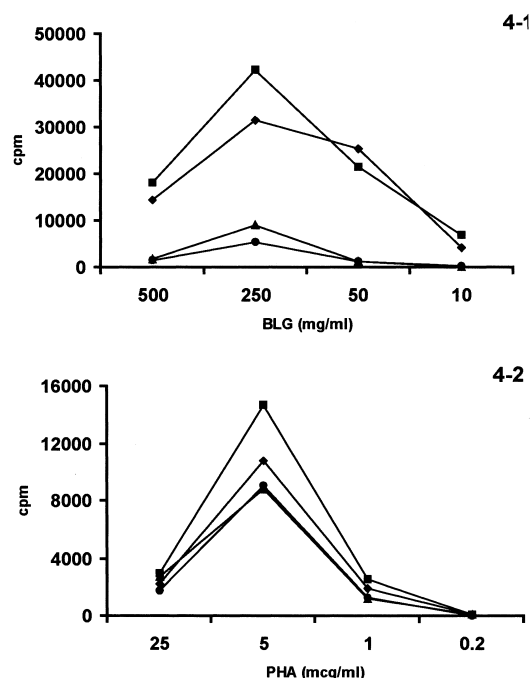


Fig. 4. Specific (1) and non specific (2) proliferative responses of splenocytes to, respectively, BLG and PHA in BALB/c mice fed either with saline (A, ■), encapsulated BLG (B4 ▲ 0.5 μ g BLG/g of body weight), soluble BLG (C1 ● 5 mg BLG/g of body weight), or blank microspheres (D, ◆).

degradable microspheres (B4) or 50 mg soluble BLG (C1), a significant decrease of DTH response was observed, with respective difference of thickness of 0.062 ± 0.024 (B4) and of 0.068 ± 0.039 (C1), compared to the difference measured in saline fed mice, 0.114 ± 0.03 (A) ($p < 0.05$).

Just as for the antibody response, both optimal oral doses of either BLG-loaded microspheres or soluble BLG were able to induce suppression of the specific DTH response. To the contrary, oral tolerance at the cellular level could not be induced for the other groups. The DTH responses of mice fed either the highest dose of BLG loaded microspheres (B1), the lowest dose of soluble BLG (C2), or unloaded microspheres (D) were not significantly different from control mice (A).

Proliferative responses to β -lactoglobulin and to Phytohaemagglutinin A have been tested on splenocytes from groups A, B4, C1, and D (Fig. 4). Hyporesponsiveness of splenic lymphocytes in both groups of mice fed optimal doses of either BLG-loaded microspheres (B4) or soluble BLG (C1) have been demonstrated (Fig. 4(1)). For both groups B4 and C1, specific proliferation to BLG was clearly decreased when compared to both saline fed mice (A) and mice fed unloaded microspheres (D). The stimulation indices to BLG, calculated at the proliferation peak data point (250 mg/ml) as the ratio of test and control ^3H thymidine incorporation values, were 0.33 and 0.44 for groups B4 and C1 respectively. Additionally, there was no significant difference between groups in levels of proliferation due to the non-specific antigen PHA (Fig. 4(2)).

4. Discussion

Our results showed that β -lactoglobulin loaded microspheres are potent tolerogens inducing specific systemic and mucosal oral tolerance in BALB/c mice. In addition, a highly significant decrease of the oral tolerogenic dose of β -lactoglobulin was obtained with oral feeding of BLG loaded microspheres. The rationale for antigen encapsulation was that this antigen preparation would offer the possibility both of protecting the tolerogen from proteolysis and of mastering the dose orally administered.

In developed countries, atopic disorders, including food allergies, have become more prevalent, in the past few decades [17]. Among the different treatments proposed to overcome these diseases, induction of specific oral tolerance against food allergens is seen as a promising tool of prevention [18]. In this perspective, oral tolerance has been well established in animal models for several different food antigens, such as OVA [19], cow's milk proteins [12,20], or gliadin [21]. Recent stu-

dies emphasise oral tolerance mechanisms and improvements for a good applicability of this inducible phenomenon [22–24].

In an attempt to modulate this tolerogenic response, namely either to decrease the efficient oral dose or to enhance the tolerisation effect, different directions have been recently considered, such as, for instance, the oral administration of beta-interferon [25]. The use of some adjuvants, well known from oral vaccine research, such as bacterial toxins : Cholera toxin (CT) and its subunit B (CTB) [26–28], or an *Escherichia coli* enterotoxin [29] have also been explored.

Among adjuvant benefits, control of the antigen delivery could be considered as a major key of success, considering the well described dose dependency in oral tolerance [6]. For this purpose, controlled administration of oral tolerogens would be greatly facilitated by antigen delivery systems, previously studied for oral vaccine development. Briefly, the advantages of antigen vectorisation include antigen protection from acidity and proteolysis in the digestive tract, as well as long and slow antigen release [30]. Particles are able to target the M cells within the Peyer's patches and are rapidly disseminated through the mesenteric system to be taken up by antigen presenting cells with enhancement of phagocytosis [31,32]. Multiple emulsion system (MES) has been previously tested for oral tolerance induction with different antigens. Paradoxically, the ability and inability of loaded MES to induce tolerance have both been conclusively assessed.

On one hand, Elson et al., have encapsulated a variant of the protein PhoA, constructed from the alkaline phosphatase of *E. coli* and observed that specific serum IgG was suppressed to a greater extent when the mice were fed the encapsulated versus the soluble protein, demonstrating the capacity of such emulsions to enhance the magnitude of oral tolerance. A similar hyporesponsiveness was obtained when bovine serum albumin (BSA) or OVA was encapsulated [33]. The present results corroborate these findings and indicate that oral tolerance can be successfully achieved in mice by feeding encapsulated food proteins.

On the other hand, the study of Flanagan, et al., should be taken into account, which showed that ovalbumin encapsulated into microparticles broke oral tolerance in mice and initiated a powerful immune response, resulting in Th2 cell activation [34]. This fact reintroduces the crucial role of the oral dose in the oral tolerance phenomenon. In their study, mice were fed 5 mg of encapsulated OVA, whereas other authors initiated the process with only a few micrograms of encapsulated proteins. Their results could be linked to the similar incapacity of our mice, fed 5 mg of encapsulated BLG (B1) to mount a tolerogenic response. Oral tolerance dose dependency is known to be essential [35] and indeed to observe a significant decrease of

specific antibodies, as well as DTH suppression, a high oral dose of soluble BLG is required in our mouse model. In a previous study, we fed mice, by one single gavage, with increasing doses of soluble BLG, from 0.5 to a maximum of 12.5 mg/g of body weight and we showed that above 1.25 mg of soluble BLG/g of body weight, oral tolerance could not be induced. This data was confirmed in the present study by group C2 [12]. By using BLG loaded microspheres as the oral tolerance inducer, the same observation was made: serum and intestinal antibody suppression and DTH hyporesponsiveness could not be achieved by every single dose, but, in this case, a very low dose was required. When BLG was encapsulated in D,L-PLG microspheres, the efficient oral dose was decreased ten thousand times, compared to that of soluble BLG. This great advantage was also observed for BSA by Elson, et al. They reported that 200 micrograms of encapsulated BSA was sufficient to induce the tolerance effects that usually required milligram amounts [33]. Such a drastic decrease in the quantity of the required oral dose is a real benefit in this system of tolerogen vectorisation. The greatest potential of these adjuvants may be their use in oral tolerance induction of precious antigens, such as synthesised or purified peptides, as encapsulation of peptides can be successfully achieved in microspheres, as previously shown [36].

Finally, if this study demonstrates that oral administration of antigen loaded in D,L-PLG microparticles is able to induce oral tolerance, we are aware that some authors report data supporting the opposite phenomenon [32]. However, even non-encapsulated food antigens present in the diet have been reported to induce both oral tolerance and systemic humoral responses, depending on their denaturation status [37]. Likewise, contradictory results to those reported by Czerkinsky have been found indicating a priming of the immune system when OVA was orally administered with cholera toxin [38]. Hearn was unable to induce oral tolerance to encapsulated OVA, whereas Elson was able to show the opposite. With regard to encapsulated antigens, Hearn's conclusion should help to keep the debate open [39]. He argued soundly that the outcome of oral administration (immunity or tolerance) of encapsulated antigen depends on multiple factors still not well controlled or understood, including the properties of the antigen itself. Further studies are required in order to improve oral adjuvant administration and to unravel contradictory data.

Overall, our finding that BLG encapsulated in microspheres is an effective tolerogen broadens the potential use of such an antigen delivery system and supports their uses within the field of food allergy prevention by oral tolerance induction.

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