

Peptides obtained by tryptic hydrolysis of bovine β -lactoglobulin induce specific oral tolerance in mice

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Background: Oral tolerance against food proteins has been achieved in different animal models with use of native or moderately hydrolyzed proteins as inducers. However, native proteins remain highly allergenic, although it has been demonstrated that protein hydrolyzates and resulting peptides can lose their allergenicity.

Objective: This study was designed to evaluate the ability of β -lactoglobulin hydrolyzate and peptides to induce oral tolerance to native β -lactoglobulin and to identify tolerogenic β -lactoglobulin peptides with low allergenicity.

Methods: β -Lactoglobulin was hydrolyzed by trypsin and fractionated by ion exchange chromatography. Peptide enrichment of fractions was evaluated. Balb/c mice were fed β -lactoglobulin hydrolyzate or fractions by single gavage at day 1. Five days later animals were challenged intraperitoneally with native β -lactoglobulin. At day 27 delayed-type hypersensitivity was performed. Twenty-four hours later mice were bled, and intestinal contents and spleens were collected. Oral tolerance was measured by titrating specific IgE in sera and intestinal samples. Specific T-cell responses were analyzed by splenocyte proliferation. Antigenicity of hydrolyzate and fractions was evaluated by specific ELISA inhibition.

Results: Mice fed either β -lactoglobulin hydrolyzate or 2 fractions of the hydrolyzate were tolerized against β -lactoglobulin. Specific serum and intestinal IgE were suppressed. Delayed-type hypersensitivity and proliferative responses were inhibited. One tolerogenic fraction was found to be 50 times less antigenic than the total β -lactoglobulin hydrolyzate was.

Conclusion: These findings support the strategy of inducing oral tolerance in "at-risk" patients by means of tolerogenic cow's milk peptides or hydrolyzate. (*J Allergy Clin Immunol* 2000;105:514-21.)

Key words: β -Lactoglobulin, cow's milk allergy, IgE, allergenicity, cow's milk protein hydrolyzate, hypoallergenicity, oral tolerance, trypsin, tryptic peptides

Abbreviations used

DTH:	Delayed-type hypersensitivity
(³ H)Tdr:	Tritiated thymidine
KLH:	Keyhole limpet hemocyanin
β -LG:	β -Lactoglobulin
OVA:	Ovalbumin
TTH β -LG:	Total tryptic hydrolyzate of β -lactoglobulin

During the common process of nutrition, dietary proteins are presented to the immune system through the gut, followed by an immune unresponsiveness to the ingested nutrients. This vital phenomenon, called oral tolerance, is efficient for the majority of people.¹ If oral tolerance fails, food allergy occurs, requiring the strict avoidance of the incriminated food.² Targeted food avoidance represents a hard task for adult patients with food allergy. To strictly remove cow's milk from the diet of allergic infants might be even more difficult, especially if breast-feeding is not possible or desired. Research in allergy prevention and treatment is particularly active because it was established that the prevalence of allergic diseases, including food allergy, has increased in the last few decades in Western countries.³ In fact, the risk of food allergy increases with family history of atopy.^{4,5} In such families prevention of cow's milk allergy is highly promoted by pediatricians.

For cow's milk allergy prevention in "at-risk" babies who cannot be breast-fed, hypoallergenic formulas are usually prescribed.⁶ In contrast to adapted formulas, cow's milk proteins have been hydrolyzed in hypoallergenic formulas to decrease the potential allergenicity.^{7,8} This approach has been demonstrated to be efficient to prevent sensitization by native proteins present in the adapted formulas.⁹⁻¹¹ Mechanisms behind this prevention may be linked to either passive prevention (avoidance of sensitizing epitopes) or active induction of oral tolerance or even to both phenomena.

Induction of such a specific oral tolerance by tolerogenic peptides derived from protein hydrolysis is seen as a potential tool to drag the immune system toward tolerization instead of sensitization.¹² However, only a few studies have been carried out to evaluate the capacity of denatured food proteins, their proteic fragments, or peptides to induce oral tolerance. It has been previously

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demonstrated in mice that heat-denatured whey proteins kept their capacity to induce oral tolerance.¹³ We have also recently shown in a rat model that a partially hydrolyzed cow's milk formula containing small- and medium-sized cow's milk protein peptides is able to induce oral tolerance to β -lactoglobulin (β -LG), whereas an extensively hydrolyzed formula does not.¹⁴

Subsequent to these previous data, the overall goal of the current study was to identify active tolerogenic peptides obtained after tryptic hydrolysis of the purified lactoglobulin. Among the cow's milk proteins, β -LG is known to be one of the major allergens. We have previously studied in mice the induction of oral tolerance to lactoglobulin by feeding the native protein.¹⁵ In the current study, according to the lower allergenicity of hydrolyzed proteins, we wished to examine the ability of a total tryptic hydrolyzate of β -LG (TTH β -LG) and of the different peptide fractions of this hydrolyzate to induce specific oral tolerance to the native protein.

METHODS

Animals

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

Antigens

Ovalbumin (OVA) (grade V) was obtained from Sigma (St Louis, Mo) and β -LG from Besnier Bridel Alimentation (Retiers, France) (β -LG acid batch 751). β -LG represented 95% of total proteins. Trypsin (EC: 3.4.21.4, bovine pancreas, 120 200 U benzoyl-arginine ethyl ester per milligram) (N-tosyl-L-phenylalanine-chloromethyl-ketone) was purchased from Sigma. A total of 220 g of β -LG dissolved in double-distilled water at 5% (wt/wt) was digested by TPKC-treated trypsin with use of an enzyme/substrate ratio of 1:100 (wt/wt) at 40  C, pH 7.6. After 1 hour of hydrolysis the same amount of enzyme was added to give a 2:100 (wt/wt) final enzyme/substrate ratio. After 4 hours of hydrolysis the reaction was stopped by inactivation of the trypsin at 85  C for 5 minutes. The digested β -LG products were separated by preparative chromatography on an anion exchange column filled with 1250 mL of source 15Q resin (Pharmacia Biotech) connected to a Biopilot system controlled by a Unicom station (Pharmacia Biotech). Samples were prepared by dissolving 20 g of TTH β -LG powder in 1400 mL of double-distilled water. The pH was adjusted to 8 by 1N sodium hydroxide. After sample injection, the column was washed by 1200 mL of solvent A (20 mmol/L TRIS-hydrochloric acid, pH 8.0). Elution was performed according to the following 2 stages of solvent B (20 mmol/L TRIS-hydrochloric acid, pH 8.0, plus 500 mmol/L sodium chloride): 0% to 40% solvent B by use of 2400 mL, then 40% to 100% by use of 3000 mL. Absorbance was recorded at 214 nm. Fifteen different fractions were obtained during this preparative chromatography. All were individually nanofiltered to be concentrated and diafiltered to eliminate salts on 20072 DDS ultrafiltration modules fitted with 20 membranes NF45. All of them were then dialyzed 3 times in 10 volumes of distilled water with SpectralPor membranes MWCO 1000 (Spectrum, Gardena, Calif), lyophilized, and dry stored at room temperature until *in vivo* experiments. Each fraction was further characterized by its peptide content with use of reverse-phase HPLC as previously described.¹⁶ Considering TTH β -LG as reference, enrichment and impoverishment of peptides in each fraction was appreciated by an area detected at 214 nm with isonitrogenous injections.

Oral tolerance induction and immunization procedures

Oral gavage was administered to the mice at age 22 days by gastric feeding of native β -LG (5 mg/g body weight), various amounts of TTH β -LG, or various amounts of the different β -LG peptic fractions. Control mice were fed saline water. Five days later, all mice were immunized by intraperitoneal injection of 0.08 mg of β -LG and 0.08 mg of OVA diluted in 0.04 mL of sterile saline solution mixed with 0.16 mL of 2% aluminum hydroxide (Superfos Biosector A/S, Denmark). Twenty-one days after systemic challenge, mice were immunized for delayed-type hypersensitivity (DTH) evaluation by intradermic injection of the left rear footpad with 0.1 mg of β -LG diluted in 0.05 mL of sterile saline solution.

Evaluation of the immune response

Twenty-four hours after DTH immunization, individual increases in footpad thickness were measured with a dial gauge microcaliper (Mitutoyo, MFG, Japan). Then mice were bled by aortic puncture under 3% isoflurane anesthesia (Abbott SA). Immediately after death, spleens were taken and pooled according to group of treatment in 20 mL of chilled RPMI 1640 (Gibco) completed with 5% FCS (Biocconcept) for specific proliferation assays. Intestinal contents were individually collected and final intestinal fluid sample dilutions were considered at 1:5, as described.¹⁷ Specific IgE against β -LG and against OVA were determined both in serum and intestinal samples.

Specific IgE antibody assays

Threefold serum dilutions and 2-fold intestinal fluid dilutions were assayed in duplicate for anti- β -LG and anti-OVA IgE antibodies by ELISA, as previously described.¹⁵ Pooled samples from 20 nonimmunized female mice were used as negative controls on each plate. Titers were determined by calculating the dilution of the sample, which gave twice the absorbance of the negative control. Titers were expressed as the log₁₀ of the reciprocal of the dilution.

Cell cultures

Spleen cell solutions were homogenized through a cellular sieve (Falcon) and purified from red blood cells by Histopaque density centrifugation (Sigma) at 390g for 20 minutes. In 96-well microtiter plates (Costar), cells (5×10^6 cells/mL) were cocultured in complete RPMI 1640 with 10% FCS in the presence or absence of either β -LG (5-0.1 mg/mL) or PHA (250 to 2 μ g/mL). Five days later, tritiated thymidine ([³H]Tdr) (Amersham, Zurich, Switzerland) was added for the final 6 hours of culture and the plates were harvested and analyzed by scintillation counting (TopCount, Canberra Packard, Zurich, Switzerland). Stimulation index values were calculated as the ratio of blank-subtracted test and control values were expressed as the mean counts per minute of (³H)Tdr incorporation by triplicate cultures.

Determination of β -LG residual antigenicity

An ELISA inhibition was used to determine β -LG IgG binding epitopes in β -LG hydrolyzate and in β -LG fractions. Briefly, Greiner microtiter plates were coated overnight with 150 μ L per well of β -LG solution (0.05 mg/mL in carbonate-bicarbonate buffer). Plates were washed with PBS-Tw and saturated for 1 hour by 0.2 mL per well of 0.5% gelatin in PBS-Tw. In separated tubes 0.2 mL of TTH β -LG or β -LG fraction samples were incubated with 0.2 mL of a 1:80,000 diluted rabbit anti- β -LG serum (produced in our laboratory) for 1 hour before the mixture was added to the plates (100 μ L/well). After 2 hours of incubation, plates were washed and 0.1 mL per well of goat antirabbit IgG labeled with horseradish peroxidase (Sigma) was added for 1 hour of incubation.

Peptide T1 (139-141)	H ₂ N-A-L-K-COOH	Peptide T2 (146-148)	H ₂ N-H-I-R-COOH
Peptide T3 (136-138)	H ₂ N-F-D-K-COOH	Peptide T4 (71-75)	H ₂ N-I-I-A-E-K-COOH
Peptide T5 (62-69)	H ₂ N-E-N-G-E-C-A-Q-K-COOH	Peptide T6 (84-91)	H ₂ N-I-D-A-L-N-E-N-K-COOH
Peptide T7 (9-14)	H ₂ N-G-L-D-I-Q-K-COOH	Peptide T8 (142-145)	H ₂ N-A-L-P-M-COOH
Peptide T9, T10, T11 (61-67,-68,-69)	H ₂ N-W-E-N-G-E-C-A,-Q,-K,-COOH		
Peptide T12 (125-135)	H ₂ N-T-P-E-V-D-D-E-A-L-E-K-COOH	Peptide T13 (1-8)	H ₂ N-L-I-V-T-Q-T-M-K-COOH
Peptide T14 (142-148) = T8 (142-145) + T2 (146-148)	H ₂ N-A-L-P-M-H-I-R-COOH	Peptide T15 (78-82)	H ₂ N-I-P-A-V-F-K-COOH
Peptide T16 (15-20)	H ₂ N-V-A-G-T-W-Y-COOH	Peptide T17 (92-99,100)	H ₂ N-V-L-V-L-D-T-D-Y-K,-K-COOH
Peptide T18 (125-138) = T12 (125-135) + T3 (136-138)	H ₂ N-T-P-E-V-D-D-E-A-L-E-K-F-D-K-COOH		
Peptide T19 (25-40)	H ₂ N-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH		
Peptide T20, T21 (61, 62-69):S-S:(149-162)	H₂N-W,-E-N-G-E-C-A-Q-K-COOH H₂N-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH		
Peptide T22 (21-40)	H ₂ N-S-L-A-M-A-A-S-D-I-S-L-L-D-A-Q-S-A-P-L-R-COOH		
Peptide T23 (41-60)	H ₂ N-V-Y-V-E-E-L-K-P-T-P-E-G-D-L-E-I-L-L-Q-K-COOH		
Peptide T24 (149-162)	H ₂ N-L-S-F-N-P-T-Q-L-E-E-Q-C-H-I-COOH		

FIG 1. Primary sequences assigned to tryptic peptides identified in β -LG hydrolyzate. Vertical bar, Disulfide bond. Sequence (102-124) was not identified.

tion. *o*-Phenylenediamine substrate was then added (100 μ L/well) for 15 minutes, and the enzymatic reaction was then stopped by addition of 25% hydrogen sulfate. Optical densities were measured at 492 nm with use of a Dynatech MR500 ELISA reader (Dynatech). β -LG binding amounts were calculated from a 5-fold dilution β -LG inhibition standard curve run on each plate (100 μ g to 1.28 ng of β -LG/mL).

Statistical analysis

DTH responses and serum and intestinal IgE responses were compared with use of ANOVA single-factor tests.

RESULTS

Characterization of fractions obtained from β -LG tryptic hydrolysis

To isolate the tolerogenic peptides from the TTH β -LG, large amounts of β -LG tryptic fractions have been produced

by 11 runs of preparative chromatography. From each of them, 15 fractions (F1-F15) were collected and respectively pooled. The assigned sequences of the β -LG tryptic peptides have been previously determined (Fig 1). However, it is of note that this set of peptides did not cover the entire amino acid sequence of bovine β -LG because the peptide corresponding to the sequence (102-124) remained unidentified. The knowledge of β -LG tryptic peptide sequences allowed the characterization of the peptide profile for each of the 15 fractions. Exhaustive analysis of the fractions showed that, except for the missing peptide (102-124), each β -LG peptide could be identified in one or several fractions (data not shown). For screening purposes, it was decided that for each fraction any enrichment of peptide equal to or greater than 3 times its respective concentration in the TTH β -LG would be considered as significant. According to this criteria, no specific peptide enrichment could be detected in

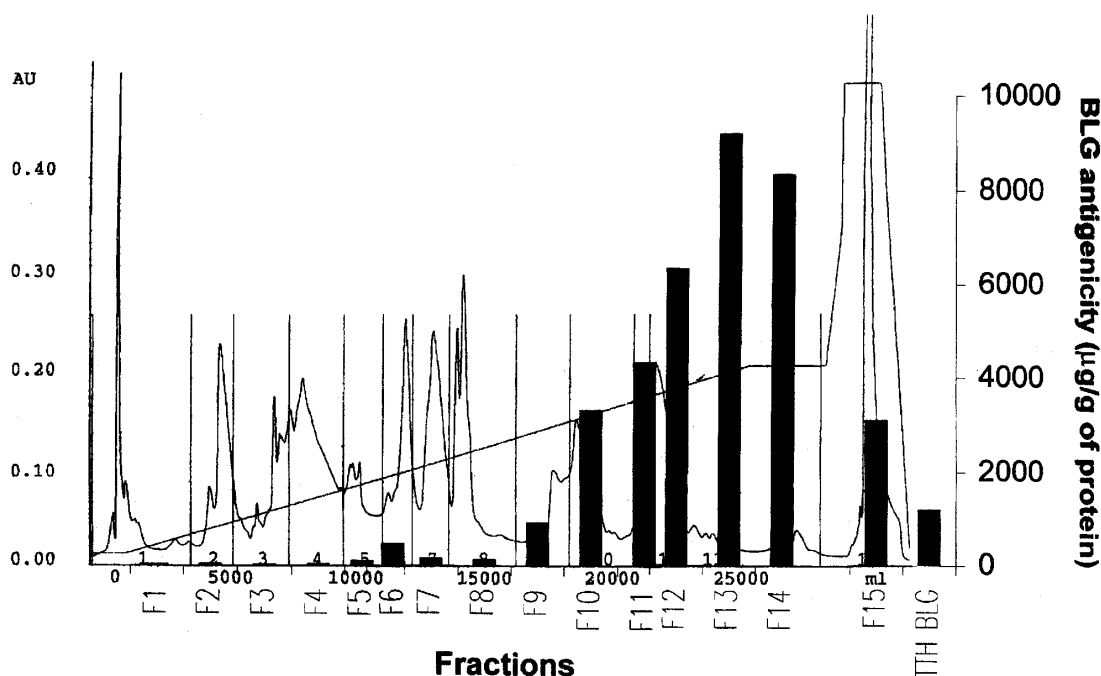


FIG 2. Antigenicity of β -LG tryptic fractions and of TTH β -LG (dark bars) reported on anionic exchange chromatogram of total tryptic hydrolyzate of β -LG on Source 15 Q Bio-Pilot column. Antigenicity corresponds to β -LG IgG-binding capacity. It is expressed in β -LG μ g/g of proteins.

fractions F1, F5, or F15. Three different peptides present in fraction F2 were enriched: T6 by 6 times, T17 and T18, eluted together, by 9 times. None of these peptides was concentrated in any of the other fractions. Similarly, T7 was specifically enriched 12 times in fraction F6. Apart from these peptides concentrated in only one given fraction, some others were enriched in several fractions. This was the case for T21: clearly detected at high amounts in fraction F7 enriched 6 times and more than 9 times in fractions F9 and F10, likewise for T23 in fractions F3 and F4. Finally, it was noticed that T10 and T11 concentrations were particularly high in 5 different fractions, from F10 to F14, enriched up to 53 times in fraction F12. Therefore the fractions were regrouped according to peptide similarities and then tested *in vivo*.

Antigenicity of the β -LG hydrolyzate and β -LG fractions

Besides peptide enrichment, measurement of residual β -LG epitopes was performed for the 15 different fractions by ELISA inhibition. It appeared that amounts of β -LG epitopes present per gram of protein ($N \times 6.38$) were highly different from one fraction to another, ranging from 15 μ g/g of protein for F1 up to 9240 μ g/g of protein for F13. Antigenicity of the fractions eluted before F10 was, however, lower than that of the total hydrolyzate. Furthermore, by superimposing the fraction antigenicity data on the TTH β -LG chromatogram, it appears that the antigenicity clearly increased from the elution of this fraction F10, with the highest rates given to the more retained acidic fractions (Fig 2).

Humoral and mucosal β -LG-specific IgE suppression

Dose-response experiments were first assayed to define the efficient oral dose of TTH β -LG (data not shown). It was found to be 2.5 mg/g mouse body weight of TTH β -LG. Results presented in Fig 3 show the achievement of oral tolerance induction with this optimal oral dose in our experimental mouse model. In this model induction of oral tolerance by native β -LG has been performed as a positive control for all the different experiments. TTH β -LG was able to induce specific humoral and mucosal tolerance to β -LG. With TTH β -LG used as the inducer, only half the required quantity of native β -LG, 5 mg/g body weight β -LG, was enough to produce this phenomenon. Suppression of both serum and intestinal anti- β -LG IgE was significant given the respective following titers: 2.68 ± 0.05 and 1.66 ± 0.32 for TTH β -LG versus 4.16 ± 0.21 and 2.32 ± 0.16 in saline solution control ($P < .05$).

To test the activity of the β -LG peptides, the 15 fractions were first individually tested *in vivo*. Mice were arbitrarily fed with 0.5 mg/g body weight of fraction. No modulation of specific humoral and cellular responses could be significantly achieved against β -LG (data not shown). To improve the screening, fractions were pooled according to their similar peptide enrichments and in proportion to their respective masses. Briefly, F1, F2, F5, and F15 were considered to be fed individually because they did not share peptide enrichments with the other fractions. F3 and F4 were pooled on the basis of a high

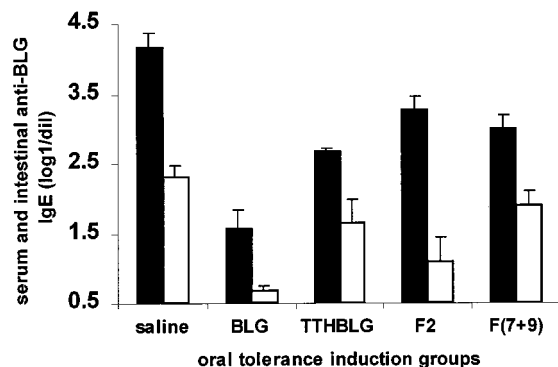


FIG 3. Humoral (dark bars) and intestinal (white bars) anti- β -LG IgE response in mice fed with either native β -LG (5 mg/g of body weight), TTH β -LG (2.5 mg/g of body weight), F2 or F(7+9) (0.125 mg/g of body weight). Control mice were fed with saline solution. Five days after feeding, mice were intraperitoneally challenged with 80 μ g of β -LG + 80 μ g of OVA. Blood and intestinal fluids were obtained 28 days after gavage.

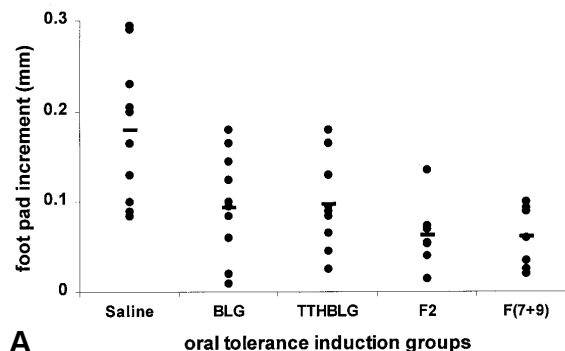
concentration of T23; F8 and F9 were mixed to test the tolerogenic activity of T12; F7 and F9 were pooled according to enrichment of T20 and T21; F6, F11, F12, F13, and F14 were pooled together, each enriched in T9, T10, and T11.

Two of these 8 tested fractions appeared to be active when given orally at 0.125 mg/g body weight: F2 and F(7+9) gave a significant suppression of serum anti- β -LG IgE (3.39 ± 0.18 vs 4.16 ± 0.21 and 3.01 ± 0.2 vs 4.16 ± 0.21 , respectively) (Fig 3). Specific intestinal IgE was also decreased in mice fed TTH β -LG (1.66 ± 0.32) or fed the 2 β -LG tryptic fractions (1.1 ± 0.34 and 1.9 ± 0.21 , respectively, for F2 and F[7+9]), assessing the orally induced down-regulation compared with the control group (2.32 ± 0.16) (Fig 3). F1 was able to induce some β -LG-specific IgE suppression, but the optimal oral dose could not be well defined. No suppression of the specific IgE antibody response could be obtained from mice fed F(3+4), F5, F(8+9), or F(6+11+12+13+14). Antigen specificity of oral tolerance induction has been determined by measuring anti-OVA IgE in parallel to anti- β -LG IgE. Immune responses to OVA were not modified by β -LG oral tolerance induction. Anti-OVA IgE titers were similar in β -LG-, TTH β -LG-, or fraction-fed groups, whether test or control groups (data not shown).

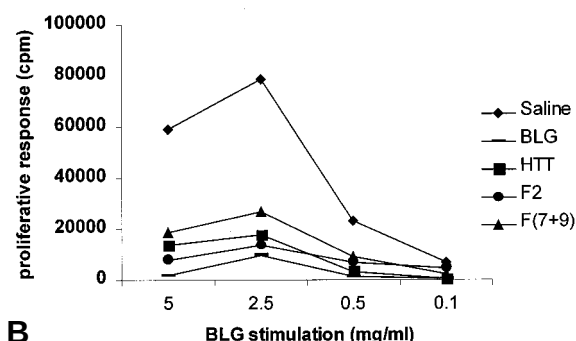
Specific suppression of cellular immune response

To confirm oral tolerance status, DTH and cell proliferation tests were also performed. DTH was significantly decreased in mice fed either native or hydrolyzed β -LG, respectively, 0.094 and 0.098 mm in comparison to local hypersensitivity measured in control mice fed only with saline solution (0.179 mm) ($P < .05$) (Fig 4, A).

Cellular tolerance has been verified as well in mice fed either with F2 or with F(7+9). Fig 4, A, shows that specific DTH was significantly reduced in mice fed with each of the 2 tolerogenic fractions 0.063 mm and 0.062 mm, respectively, for F2 and F(7+9) in comparison to the



A



B

FIG 4. Specific cellular oral tolerance induction in mice fed once with either β -LG (5 mg/g body weight), TTH β -LG (2.5 mg/g body weight), or F2 or F(7+9) (0.125 mg/g body weight). Control mice were fed with saline solution. Five days after feeding, mice were intraperitoneally challenged with 80 μ g of β -LG + 80 μ g of OVA. DTH tests were performed 27 days after gavage, by intradermal injection of 100 μ g of β -LG in the rear left footpad. Measurements of footpad thickness were done before injections and 24 hours later. Mice were killed and spleen cells were obtained 28 days after gavage. **A**, β -LG-specific DTH response (solid circles) are individual footpad increments, and horizontal lines are means from 10 mice per group. **B**, Specific proliferative responses of splenic cells. Splenic lymphocytes from mice fed with either saline solution (solid diamonds), β -LG (horizontal lines), TTH β -LG (HTT) (solid squares), F2 (solid circles), or F(7+9) (solid triangles) were isolated and subsequently stimulated with decreasing concentrations of antigens. (^{3} H)Tdr incorporation was measured after 120 hours of culture. (^{3} H)Tdr incorporation results were expressed in counts per minute, as a mean of triplicate cultures; the blank-subtracted mean was then plotted respectively against β -LG concentration.

footpad thickness (0.179 mm) observed in control mice ($P < .05$).

On the other hand, β -LG-specific proliferation of splenocytes isolated in TTH β -LG- and in β -LG-fed mice was reduced to 0.225 and 0.120, as respective stimulation index values (Fig 4, B). Fig 4, B, also shows that feeding mice either of the 2 elected β -LG fractions as well as TTH β -LG provoked a severe decrease of the proliferative response to β -LG in comparison to the one observed from splenocytes isolated from saline solution-fed control mice. Indeed, stimulation index values were 0.174 and 0.341, respectively, for F2- and F(7+9)-fed mice. The proliferative response of splenocytes from mice fed the other peptide fractions were not reduced,

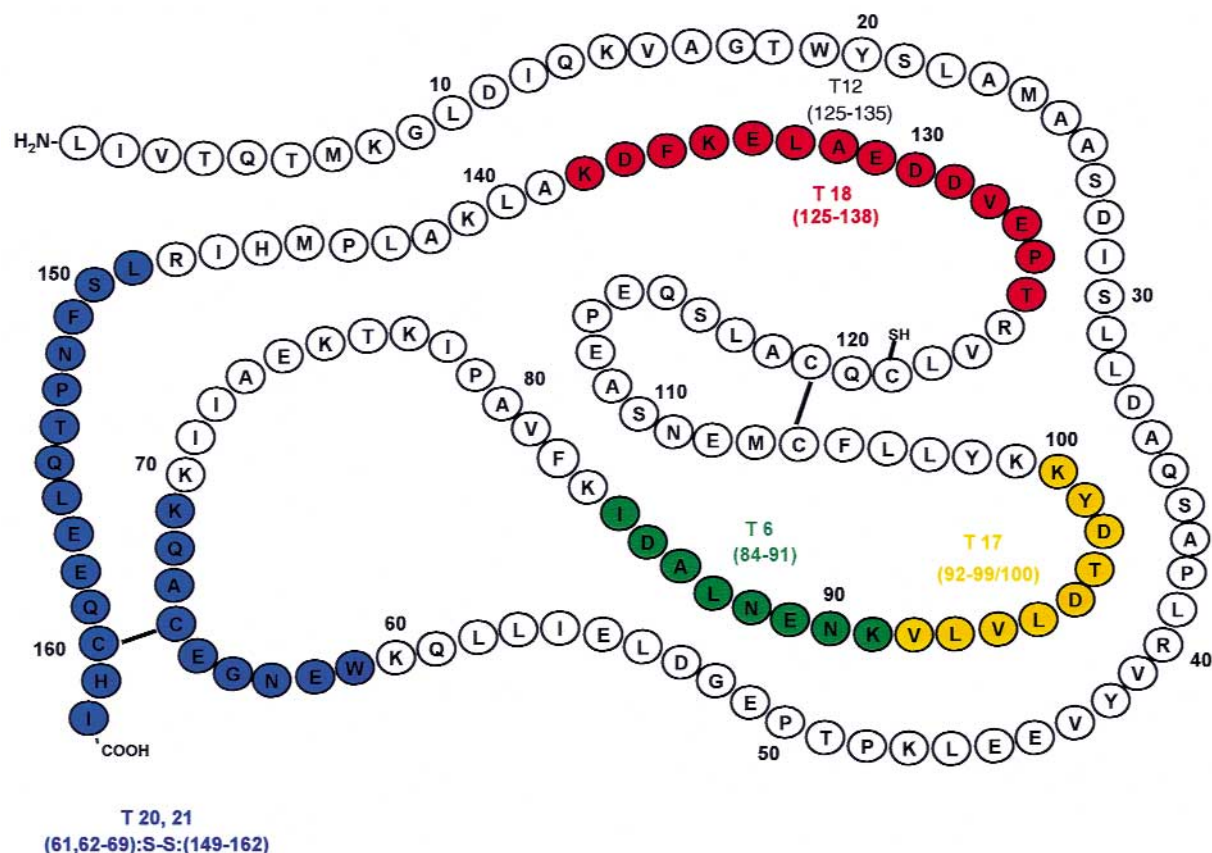


FIG 5. Tolerogenic tryptic peptides location on primary sequence of native bovine β -LG. Peptides T6, T17, and T18 were enriched in F2. Peptides T20 and T21 were enriched in F(7+9).

with the exception of the group fed with F(8+9), for which the stimulation index was low (0.29). As expected, the nonspecific proliferative response to PHA was not affected by the different feeding regimens; for all groups it remained unmodified between the fed and control groups (data not shown).

Localization of the tolerogenic peptides enriched in fractions F2 and F(7+9) on the primary sequence of β -LG

The tryptic peptides found to have tolerogenic properties have been located on the primary sequence of native bovine β -LG, as presented on the schematic model of the molecule (Fig 5). The tolerogenic fraction F2 was enriched for the 3 tryptic peptides located on the inside of the schematic representation of the β -LG molecule, from the 84th to the 100th amino acids, corresponding to peptides T6, T17, and from the 125th to the 138th amino acids, for T18. On the other hand, F(7+9) also contained T12, derived from T18, but was mainly enriched with peptides T20 and T21, (61,62-69):S-S:(149-162), located at the carboxylic extremity of the molecule, including 1 disulfide bond.

DISCUSSION

Our results showed that β -LG hydrolyzate and β -LG peptides can induce oral tolerance to native intact β -LG at the humoral and cellular levels. Mice fed the optimal dose of TTH β -LG achieved oral tolerance, fulfilling our 3 criteria of hyporesponsiveness. Furthermore, 2 β -LG tolerogenic fractions, F2 and the mixture F(7+9), were also found capable of inducing oral tolerance to native β -LG. In these 2 tolerogenic fractions the efficient oral dose as well as the size and structure of the peptides appeared to be crucial.

With respect to the efficient oral dose, a dose dependency of oral tolerance induction was observed, as previously described.¹⁸ In comparing the 2 tolerogenic fractions, it was also interesting to note that both hydrolysis and peptide enrichment resulted in a decrease in the efficient oral dose. Besides targeting the tolerance induction without the risk of sensitization, the use of tolerogenic peptides caused a dramatic decrease in the efficient dose of potent material. Therefore it is conceivable that purified β -LG tolerogenic peptides will require only a few micrograms to be active, just as already seen in an oral tolerance experimental model using peptides as for

instance from *Escherichia coli* enterotoxin.¹⁹ This hypothesis is being investigated in our mice model.

In consideration of the peptide size, the lengths of the potentially tolerogenic peptides were distributed between 8 amino acids for T6 and 23 amino acids for T21, given respective molecular weights between 915 and 2719 d.¹⁶ Our results showed that the tolerogenic β -LG peptides were all of average size less than 4500 d. Hashimura et al²⁰ have reported similar oral tolerance induction by feeding mice with a tryptic digest of casein in which peptide fragments were smaller than 6000 d. Michael²¹ had the same approach, hydrolyzing BSA and feeding mice with the BSA-hydrolyzed fractions. Oral administration of BSA peptide fraction, exhibiting a molecular size of about 13,000 d, did tolerize mice against native BSA. Our data then are consistent with the sizes of purified or synthesized tolerogenic peptides previously reported in the literature, given tolerogenic peptide sizes around 20 amino acids.^{19,22,23}

Finally, with respect to the peptide structure, we knew that the tolerogenic F(7+9) differed from the other fractions containing T20 and T21 mainly by the presence of a disulfide bond between peptides T20 and T21. We did observe, by chemically reducing the tolerogenic fraction F(7+9), that the tolerogenic property of this fraction was abolished (data not shown).

To be tolerogenic, the peptides seem to require a precise balance among size, sequence, structure, and dose. However, until now correlation between peptide structure and tolerogenic properties has not been described in the literature. This fundamental link between structure and function still remains to be explored to unravel oral tolerance induction by peptides.

In parallel to their tolerogenic ability, the antigenicity of the tolerance inducers has been studied. Induction of oral tolerance with hydrolyzed proteins may present 2 major pitfalls: first, the sensitization of the host by the tolerogenic inducer, if allergenicity remains, or, contrarily, the lack of preventive tolerization, if an innocuous, but, impotent tolerogenic inducer obtained after a drastic enzymatic digestion is used. Thus tolerogen preparation appears to be a meticulous task in which hydrolysis is a key step. In the current study the hydrolysis conditions, enzyme, pH, and temperature, were selected to provoke a mild digestion of β -LG because we knew from a previous experiment that mice fed an extensively hydrolyzed formula were unable to mount a tolerogenic response to β -LG (data not shown). The measures of residual antigenicity indicate that antigenic and tolerogenic sites might be distinctly located, suggesting that allergenicity and tolerogenicity could be uncoupled. Our data corroborate those of a previous study of the antigenic properties of β -LG suggesting the discontinuous feature of the antigenic structure of the molecule.²⁴ Schematically, 3 different types of fractions can be described in our model: a first type (F2), including basic peptides with a high tolerogenic potential associated with a very low antigenicity; at the opposite, a fraction type containing acidic peptides, highly antigenic and devoid of tolerogenic

activity (F13); and in the middle, the fractions containing peptide inducers of tolerance but still having notable antigenicity (F7, F9). Because of the extremely low antigenicity of the F2, the tolerogenic peptides included in this fraction seem to be of particular interest. Thus it can be hypothesized whether T6, T17, or T18 peptides, all of which are present in fraction F2, would be tolerogenic if administered orally. This hypothesis will be further tested to improve tolerogen identification.

Our data strengthen the concept that specific oral tolerance induction could be initiated in at-risk newborn infants by means of hypoallergenic formulas enriched in tolerogenic peptides. Native proteins are inappropriate to be considered as oral tolerogens in at-risk newborn babies. Several clinical studies have reported the high prevalence of atopic symptoms resulting from sensitization in at-risk infants fed adapted formulas.²⁵⁻²⁷ Two different types of hypoallergenic formulas are therefore proposed for high-risk babies: partially and extensively hydrolyzed formulas. Feeding partially hydrolyzed formulas has been demonstrated to allow the induction of oral tolerance in a rat experimental model, whereas extensively hydrolyzed formulas could not.¹⁴ These results indicated that small peptides, derived from extensive hydrolysis, were not tolerogenic. A nonallergenic basis enriched in tolerogenic peptides could be seen as an intermediate approach to induce tolerance.

Another step has been passed on the way to disease prevention by oral tolerance induction. Recently, several studies have described experimental oral tolerance induction in humans by feeding volunteers with keyhole limpet hemocyanin (KLH), showing a T-cell tolerance induction at both systemic and mucosal sites²⁸ and also a down-regulation of specific immune responses to KLH.²⁹ Experimental animal data completed with these preliminary results in volunteers are encouraging. They all predict oral tolerance induction as a future preventive strategy of great potential in allergic diseases.

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