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Immunomodulatory Potential of Partially Hydrolyzed β-Lactoglobulin and Large Synthetic Peptides

Karine Adel-Patient,*[,†] Sophie Nutten,[§] Hervé Bernard,[†] Rodolphe Fritsché,[§] Sandrine Ah-Leung,[†] Narimane Meziti,[†] Guénolée Prioult,[§] Annick Mercenier,[§] and Jean-Michel Wal[†]

[†]INRA, UR496 Immuno-Allergie Alimentaire, CEA/IBiTeC-S/SPI, CEA de Saclay, F-91191 Gif sur Yvette cedex, France

[§]Nestle Research Center, Lausanne, Switzerland

ABSTRACT: The immunomodulatory potential of fragments derived from the cow's milk allergen bovine β-lactoglobulin (BLG) was assessed in a mouse model of oral tolerance (OT) [Adel-Patient, K.; Wavrin, S.; Bernard, H.; Meziti, N.; Ah-Leung, S.; Wal, J. M. Oral tolerance and Treg cells are induced in BALB/c mice after gavage with bovine β-lactoglobulin. *Allergy* **2011**, *66* (10), 1312−1321]. Native BLG (nBLG) and chemically denatured BLG (lacking S−S bridges, dBLG), products resulting from their hydrolysis using cyanogen bromide (CNBr) and some synthetic peptides, were produced and precisely characterized. CNBr hydrolysates correspond to pools of peptides of various sizes that are still associated by S−S bridges when derived from nBLG. nBLG, dBLG, and CNBr hydrolysate of nBLG efficiently prevented further sensitization. CNBr hydrolysate of dBLG was less efficient, suggesting that the association by S−S bridges of peptides increased their immunomodulatory potential. Conversely, synthetic peptides were inefficient even if covering 50% of the BLG sequence, demonstrating that the immunomodulatory potential requires the presence of all derived fragments of BLG and further supporting the use of partially hydrolyzed milk proteins to favor OT induction in infants with a risk of atopy.

KEYWORDS: *oral tolerance, immunomodulation, cow's milk allergy, β-lactoglobulin, chemical hydrolysis, synthetic peptides, partial hydrolysis*

INTRODUCTION

Oral tolerance (OT) to food proteins is a normal immune process whereby the immune system recognizes common antigens as being harmless.[1] This allows preserving the integrity of the body confronted with innocuous food antigens and microbiota while inducing an efficient defensive immune response against potentially harmful microorganisms or toxins. Food allergy, which is an increasingly prevalent immune disease with potential life-threatening manifestations, is considered to result from an impaired development or a breakdown in existing tolerance.[2]

The efficacy of OT induction depends on numerous factors such as the genetic background and age of the individual, environmental conditions, the composition of the intestinal microbiota, physicochemical characteristics of the antigen, and the route, frequency, and doses of antigen exposure. According to some studies, Peyer's patches are crucial for allowing the induction of OT[3−5] but others rather showed the critical role of antigen transport into the mesenteric lymph nodes (MLN) via dendritic cells (DC).[6] A recent study by Hadis and collaborators suggests that regulatory T cells (Treg) are induced in the MLN after migration of antigen-loaded $CD103^+$ DC from the *lamina propria* (LP).[7] The induced Treg acquire the capacity of homing to the LP of the small intestine, via the expression of integrin α4β7 and chemokine receptor CCR9,[8] and sustained oral tolerance required Treg expansion in the LP under the control of resident $CX3CR1^+$ macrophages producing IL-10.[7] The induced Treg can also disseminate in the body, allowing systemic tolerance.[7,9]

Although recently called into question,[10] certain hydrolyzed milk formulas, and notably partially hydrolyzed milk formulas, for example, in which large peptide fragments are still present, have been shown to reduce the risk of allergenicity, notably atopic dermatitis.[11,12] Such formulas are then used for primary prevention in infants with a family history of allergic disease. Extensively hydrolyzed milk formulas are considered to be efficient as a secondary prevention, to avoid allergic reaction in already sensitized children. Accordingly, several studies have demonstrated that the use of formulas made of extensively hydrolyzed cow's milk proteins from whey and/or casein fractions, containing only peptides of molecular weight lower than 5000 Da, is appropriate for cow's milk allergic patients as the proteins have lost their antigenic and allergenic potential, notably to elicit an allergic reaction.[13−17] In contrast, partially hydrolyzed formulas were shown to keep part of their antigenicity, including the capacity of IgE antibody induction,[15−18] and to keep a tolerogenic potential, whereas extensively hydrolyzed proteins did not retain such potential.[19,20] Partially hydrolyzed formulas have thus been recommended at the cessation of breast-feeding in infants with high risk of atopy. They contain protein fragments of various sizes but may also still contain residual nonhydrolyzed proteins.[20,21] Therefore, it is difficult to assess the relative role of those different products in the tolerating process and/or to identify essential specific fragments. To clarify the influence of the conditions and degree of hydrolysis, we have analyzed the immunomodulatory potential of bovine β-lactoglobulin (BLG),

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the major component of whey and of some of its products generated by chemical hydrolysis.

BLG is a globular protein of 162 amino acids (MW 18 kDa) containing two intramolecular disulfide bridges and present as a dimeric form at physiological pH values.[22,23] We have recently evidenced the efficacy of tolerance induction in wild type BALB/cJ mice by gavage with native BLG (nBLG) and demonstrated that it induced Treg at the local and systemic levels. This resulted in an efficient inhibition of further sensitization and elicitation of the allergic reaction to BLG. Conversely, gavage with nBLG hydrolyzed with trypsin, a duodenal enzyme that splits BLG in numerous small fragments (e.g., <20-mer), allowed only a local induction of Treg (i.e., in MLN) and partial inhibition of sensitization with no effect on the elicitation phase.[9] As extensive hydrolysis may destroy most of the BLG T-cell epitopes, it would extensively reduce the initiation of an immune response, whatever it is an effectory or regulatory response. In the present study, we thus aimed at analyzing the effect of gavage with chemically hydrolyzed native or denatured (i.e., in which S−S bridges are disrupted) BLG on further sensitization to BLG in mice. The hydrolysis was performed using cyanogen bromide (CNBr), which generates fewer and larger fragments than trypsin hydrolysis. In addition, two synthetic peptides covering up to 50% of BLG sequence were tested. All products were carefully characterized for purity, chemical structure, and biological activity (i.e., immunoreactivity) before administration to mice.

MATERIALS AND METHODS

Safety. All CNBr manipulations were conducted under an extractor hood by experienced personnel wearing adapted protective gloves, clothing, and security goggles. All potentially contaminated solutions were treated with 4% NaOH and 5% hypochlorite sodium solution, according to the safety data sheet from NIH and Lunn and Sansone.[24] All contaminated materials were sequentially treated with sodium hydroxide and sodium hypochlorite solutions. All wastes were then selectively removed.

Apparatus and Reagents. Enzyme immunometric assays were performed in 96-well microtiter plates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) using AutoPlate Washer and Microfill dispenser equipment from BioTek Instruments, Inc. (Avantec, Rungis, France). Unless otherwise stated, all reagents were of analytical grade from Sigma (St. Louis, MO, USA).

Preparation, Purification, and Hydrolysis of BLG and Peptide Synthesis. Native BLG (nBLG) variant A was prepared and purified from untreated raw cow's milk (Ferme de Viltain, Saclay, France) using selective precipitation and chromatography as previously described.[25,26] Chemically denatured BLG (hereafter called denatured BLG, dBLG) was obtained after reduction and alkylation of nBLG as previously described.[26,27] Purified proteins were dialyzed against potassium phosphate buffer (100 and 20 mM, pH7.4) and freeze-dried.

Cyanogen bromide (CNBr) hydrolysis of nBLG and dBLG was performed in 8 M urea and 0.1 M HCl[27−29] using a ratio of 40 mol of CNBr per mole of methionine. After 36 h at 20 °C, 10% of 1 M NaOH was added for CNBr deactivation,[24] and the excess of CNBr was removed by exclusion chromatography using a Sephadex G10 column (2.5 × 30 cm) equilibrated in 50 mM phosphate buffer, 0.5 M NaCl, pH 7.4. As CNBr hydrolysates contained about 1% of residual undegraded BLG, an additional purification step was performed by RP-HPLC using a AKTA system (GE Healthcare, France) with a Vydac C18 column (300 Å, 250 × 22 mm) and solvents A (H_2O/0.1% trifluoroacetic acid) and B (acetonitrile/0.04% trifluoroacetic acid). After a first step at 0% of solvent B, elution of all breakdown products was achieved by a step at 40% of solvent B. Residual nondegraded BLG was discarded by a final step at 44% of solvent B.

Synthetic peptides corresponding to BLG sequences (84−106) and (25−107) were synthesized using chemical coupling HOBT/DCC and Fmoc chemistry on ABI 433A peptide synthetizer (Applied Biosystems). Peptides were further purified by RP-HPLC on Vydac C18 column (250 × 22 mm) using a linear gradient of solvents A and B.

CNBr hydrolysates and synthetic peptides were freeze-dried. After solubilization in PBS (DPBS Gibco), protein/peptide content was assayed using a BCA protein assay kit from Pierce, following the manufacturer's instructions.

Characterization of Hydrolysis Products. All proteins, CNBr hydrolysates, and synthetic peptides were further characterized by electrophoresis under reducing and nonreducing conditions (Nu-PAGE system, Invitrogen, Renfrewshire, UK), immunoblot (XCell II Blot Module, Invitrogen) with a monoclonal antibody (mAb) specific for BLG region (41−60),[30,31] RP-HPLC (AKTA system), mass spectrometry (MALDI-TOF, Voyager DE-Pro, Applied Biosystems), and specific sandwich immunoassays for native and denatured BLG.[30]

The immunoreactivity of BLG-derived products and synthetic peptides was assessed using a set of mouse monoclonal antibodies (mAb) specific of different well-identified BLG peptides comprising sequential epitopes.[30,31] A competitive enzyme immunoassay was performed between BLG-derived products and BLG labeled with acetylcholinesterase (AChE-BLG)[30] for binding to the mAbs. Briefly, 50 μL of mAb, 50 μL of BLG-derived products (i.e., competitor, from 10 to 0.01 μg/mL), and 50 μL of AChE-BLG were incubated for 18 h at 4 °C on goat anti-mouse antibodies (Jackson Immuno Research Laboratories, West Grove, PA, USA) coated plates. Plates were then washed, and 200 μL of Ellman's reagent was distributed per well.[32] Absorbance at 414 nm was measured after 1−2 h of enzymatic reaction. Curves are plotted in which B/B_0 values are expressed as a function of competitor concentrations. B and B_0 represent solid-phase bound enzymatic activity measured in the presence or absence of competitor, respectively.

Mice. Specific pathogen-free (SPF) BALB/cJ mice (3−4-week-old females, Centre d'Elevage René Janvier, Le Genest Saint-Isle, France) were housed in filtered cages under normal SPF husbandry conditions (autoclaved bedding and sterile water) and were acclimated for 2 weeks before experiments. They were maintained on a diet deprived of animal proteins in which BLG was not detected using specific sandwich immunoassays.[30] All animal experiments were performed according to European Community rules of animal care and with authorization 91-368 of the French Veterinary Services. All experiments were covered by Agreement 2009-DDSV-074 (October 29, 2009) from the Veterinary Inspection Department of Essonne (France).

BLG Product Administration and Effect on a Further Sensitization of Mice by nBLG. In a first set of experiments, groups of six or seven mice were administered 2.4 mg of nBLG, dBLG, CNBr hydrolysate of native BLG (CNBr-nBLG), or CNBr hydrolysate of denatured BLG (CNBr-dBLG), all diluted in PBS. Administrations were performed on days 1, 2, 3, 8, 9, and 10 by means of intragastric gavage using an animal feeding needle (Popper & Sons, NY, USA). Control mice received PBS (negative control mice, n = 16). All mice were then sensitized on day 14 by ip administration of 5 μg of nBLG adsorbed on alum (Alhydrogel 3%, Superfos, Denmark, 1 mg/mouse).

In a second set of experiments, following the same protocol, mice were administered 4 mg of nBLG or equivalent quantity (i.e., 0.22 μmol) of isolated synthetic peptides. Either 0.6 mg of synthetic peptide (84−106) (MW 2731 Da) or 2 mg of synthetic peptide (25−107) (MW 9440 Da) was administered (n = 7/group). Nine control mice received PBS. All mice were then sensitized as described above.

The efficacy of mice sensitization was assessed by quantitative measurements of BLG-specific IgE and IgG1 antibodies[33] on individual serum samples collected from the retro-orbital venous plexus under light anesthesia (Isoflurane, Baxter) between days 30 and 33. Nonspecific binding was determined using individual sera from 10 naïve mice and was deduced of the absorbance values measured with immune sera. IgE and IgG1 antibodies against synthetic peptide (25−107) were also measured using plates coated with the corresponding

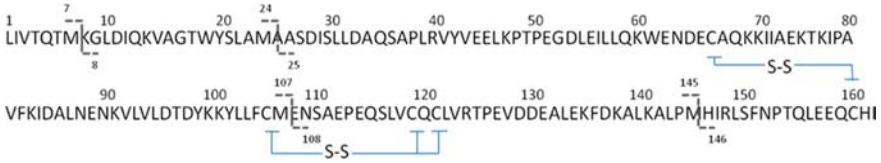

Figure 1. BLG amino acid sequence (from Swiss-Prot: P02754.3 and Sélo et al.[39]). Disulfide bridges (S−S) and CNBr cleavage sites (dashed lines) are shown.

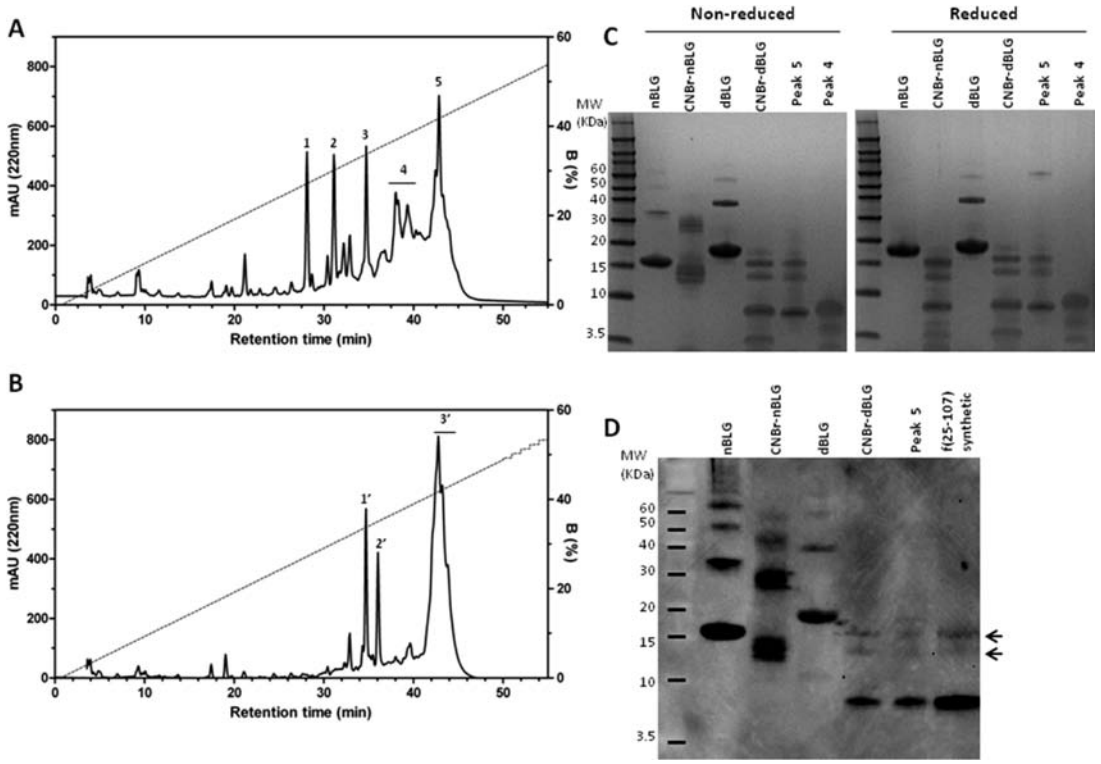

Figure 2. Biochemical characterization of CNBr hydrolysates: (A) RP-HPLC of CNBr-dBLG; (B) RP-HPLC of CNBr-nBLG (mass data obtained for major collected peaks are provided in Tables 1 and 2); (C) electrophoresis under nonreducing and reducing conditions of nBLG, dBLG, corresponding CNBr hydrolysates and peaks 5 and 4 collected from CNBr-dBLG [samples were diluted in LDS loading buffer plus reducing agent and heated for 10 min at 70 °C (reducing conditions) or were diluted in LDS loading buffer (nonreducing conditions); proteins were then loaded on NuPAGE 12% Bis-Tris Gel and run for 35 min at 200 V in MES buffer; 8 μL of Novex Sharp Standard was loaded in the first well; all reagents and materials were from Invitrogen]; (D) immunoblot of nBLG, dBLG, corresponding CNBr hydrolysates, peak 5, and synthetic peptide (25−107) [electrophoresis under nonreducing conditions was performed as in panel C; proteins were then transferred to PVDF membrane using XCell II Blot module (Invitrogen) and incubated with a mAb specific for the BLG region (41−60) (1 μ/mL); after incubation with secondary antibody (peroxidase-conjugated ImmunoPure goat anti-mouse IgG, ThermoScientific, Pierce, Rockford, IL, USA), bands were revealed using Amersham ECL Plus Western detection system (GE Healthcare, Buckinghamshire, UK)].

peptide (10 μg/mL, 0.05 M phosphate buffer, pH 7.4). After bleeding, anesthetized mice were sacrificed by vertebral dislocation, and spleens were removed under sterile conditions. Spleens were pooled within groups to evaluate cytokine production under nonspecific and specific ex vivo restimulation, as previously described.[9] IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, GM-CSF, IFN-γ, and TNF-α were assayed using BioPlex technology and mouse cytokine kits from Bio-Rad, following the provider's recommendations. BLG-specific secretion was obtained after subtraction of nonspecific secretion induced by ovalbumin. No statistical analysis was performed because results are expressed as the mean ± SEM of duplicate determinations on supernatants from spleens pooled within pretreatment groups.

Statistical Analysis. All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Normality distribution was first examined using the Shapiro−Wilk normality test before analysis of statistical significance with one-way ANOVA and Tukey's multiple-comparison post test. When data were not normally distributed, a nonparametrical test was performed, using the Kruskal−Wallis test followed by Dunn's multiple-comparison test (DMCT). Differences between groups were regarded as significant when $p < 0.05$. Comparison of two specific groups was performed using unpaired t tests with Welch's correction on log-transformed data, obtaining comparable variance.

RESULTS

Characterization of CNBr Hydrolysis Products. BLG primary sequence, location of disulfide bridges, and sequence of theoretical derived products after CNBr hydrolysis are depicted in Figure 1. CNBr hydrolysis of native BLG may lead to fragments linked by disulfide bridges between cysteines 66 and 160 and 106−119/121. CNBr hydrolysates of dBLG and nBLG were then analyzed by RP-HPLC and mass spectrometry (Figure 2, panels A and B, respectively), SDS-PAGE under nonreducing and reducing conditions (Figure 2C), and immunoblotting using a mAb specific for the BLG region (41−60) (Figure 2D). Although a faint band was visible at MW

>18000 Da in SDS-PAGE, no residual entire BLG was detected in any CNBr-derived products using specific and sensitive immunoassays (data not shown). Moreover, no peak corresponding to intact BLG, that is, RT 48 min and MW 18362 for nBLG and RT 46 min and MW 18647 for dBLG, was detected in any BLG-derived products. This band very likely corresponds to polymers of generated fragments, as observed in the electrophoretic pattern of BLG.

Mass analysis of major fractions collected from CNBr-dBLG products revealed the presence of fragment f(8−24) and alkylated fragments f(25−107), f(108−145), f(108−162), and f(146−162) (Figure 2A; Table 1). Evidence of alkylated fragment f(108−162) suggests an incomplete hydrolysis of the Met[145]−His[146] bond. Interestingly, fragment f(25−40) was also detected, suggesting nonspecific hydrolysis. Electrophoretic patterns of CNBr-dBLG and of collected fractions 5 and 4, corresponding to the major identified fragments f(25−107) and f(108−145) + f(108−162), respectively, are shown Figure 2C. Corresponding immunoblot with anti-(41−60) mAb of these entities but also of synthetic peptide (25−107) is also provided (Figure 2D, nonreducing conditions). Altogether, these data demonstrate that the most abundant fractions in CNBr-dBLG hydrolysate correspond to (i) fragment f(25−107), which migrates as three distinct bands of approximate MW 16, 13, and 9 kDa, probably as a result of dimer formation through hydrogen bonds and electrostatic interactions, notably between Asp[33] and Arg[40],[34] and (ii) fragments f(108−145) and f(108−162). All of these results were further confirmed by in-gel trypsinolysis and mass spectrometry analysis by checking for the presence of relevant reporter masses 837, 1716, 2314, and 1627 Da, corresponding to BLG sequences (142−148), (149−162), (41−60), and (25−40), respectively (data not shown).

Table 1. Measured Masses and Sequence Assignment for Major Peaks Collected from CNBr-dBLG (Figure 2A)

peak	measured mass (nonreducing conditions)	sequence assignment	theoretical mass	comments[a]
1	1628	(25−40)	1626	Cys-CAM
2	2123	(146−162)	2123	
3	1833	(8−24)	1833	
4	4315	(108−145)	4314	Cys-CAM
	6467	(108−162)	6466	Cys-CAM
5	9505	(25−107)	9506	Cys-CAM

[a]Cys-CAM, carbamidomethylcysteine.

Mass analysis of major fractions collected from CNBr-nBLG hydrolysate revealed the presence of both fragments f(8−24) and f(1−24), suggesting an incomplete hydrolysis of Met[7]−Lys[8] bond (Figure 2B; Table 2). A major broad peak corresponding to the peak eluted at 44 min in RP-HPLC (peak 3) was observed at an average mass of 15748 Da under nonreducing conditions. Analysis of this fraction by mass spectrometry under reducing conditions revealed the presence of different compounds, that is, fragments f(25−107), f(108−145), f(108−162), and f(146−162) (Table 2). The presence of the f(108−162) entity once again suggests an incomplete hydrolysis of the Met[145]−His[146] bond. Comparison of electrophoresis patterns of CNBr-nBLG and CNBr-dBLG under reducing conditions further supports this composition (Figure 2C). Moreover, SDS-PAGE of CNBr-nBLG under nonreducing and reducing conditions confirms the association of these fragments by S−S bridges. Compounds f(25−145) and f(25−162), which may result from an incomplete hydrolysis of Met[107]−Glu[108] and Met[145]−His[146] bonds, were also detected by mass spectrometry within peak 3, but with very low signals, suggesting their very low abundance in the CNBr-nBLG hydrolysate.

Altogether, these data thus suggest that CNBr hydrolysates are mainly composed of fragments f(25−107), f(108−145), and f(108−162) that are still associated by S−S bridges when derived from nBLG.

Immunoreactivity of Whole CNBr Hydrolysates and Synthetic Peptides. CNBr-nBLG, CNBr-dBLG, and the synthetic peptide (25−107) that corresponds to the largest fragment generated by CNBr hydrolysis were further analyzed using finely characterized anti-BLG monoclonal antibodies of different specificities. Inhibition of the binding of labeled BLG to mAbs specific for BLG regions (41−60) and (133−143) by nBLG, dBLG, and corresponding CNBr hydrolysates is shown in Figure 3, panels A and B, respectively, showing similar results. Competitive tests performed with nBLG demonstrated the low accessibility of these B-cell linear epitopes in the native protein when compared to dBLG. Interestingly, CNBr hydrolysates (i.e., CNBr-dBLG and CNBr-nBLG) demonstrated a similar binding capacity to those mAb, which was higher than that of nBLG, suggesting that partial hydrolysis of nBLG made B-cell epitopes more easily accessible for mAb binding. Inhibition of the binding to mAb specific for region (149−162) demonstrated that nBLG is once again less reactive than dBLG (Figure 3C). However, recognition of dBLG by this mAb is lower than that observed with antibodies specific for regions (41−60) and (133−143), reflecting lower antibody affinity and/or the fact that carboxymethylation using iodoacetamide has partly modified this mAb epitope, lowering dBLG immunoreactivity. The immunoreactivity of CNBr-dBLG was

Table 2. Measured Masses and Sequence Assignment for Major Peaks Collected from CNBr-nBLG (Figure2B)

peak	measured mass: nonreducing conditions	measured mass: reducing conditions	sequence assignment	theoretical mass	comments[a]
1′	1834		(8−24)	1833	
2′	2622		(1−24)	2620	
3′		2066	(146−162)	2066	
		4202	(108−145)	4200	
		6312	(108−162)	6311	MSO
		9395	(25−107)	9392	
		13657 (minor)	(25−145)	13654	MSO
		15823 (minor)	(25−162) ?	15750	MSO + β-MEA adducts (+76)

[a]MSO, methionine sulfoxide; β-MEA, β-mercaptoethanol.

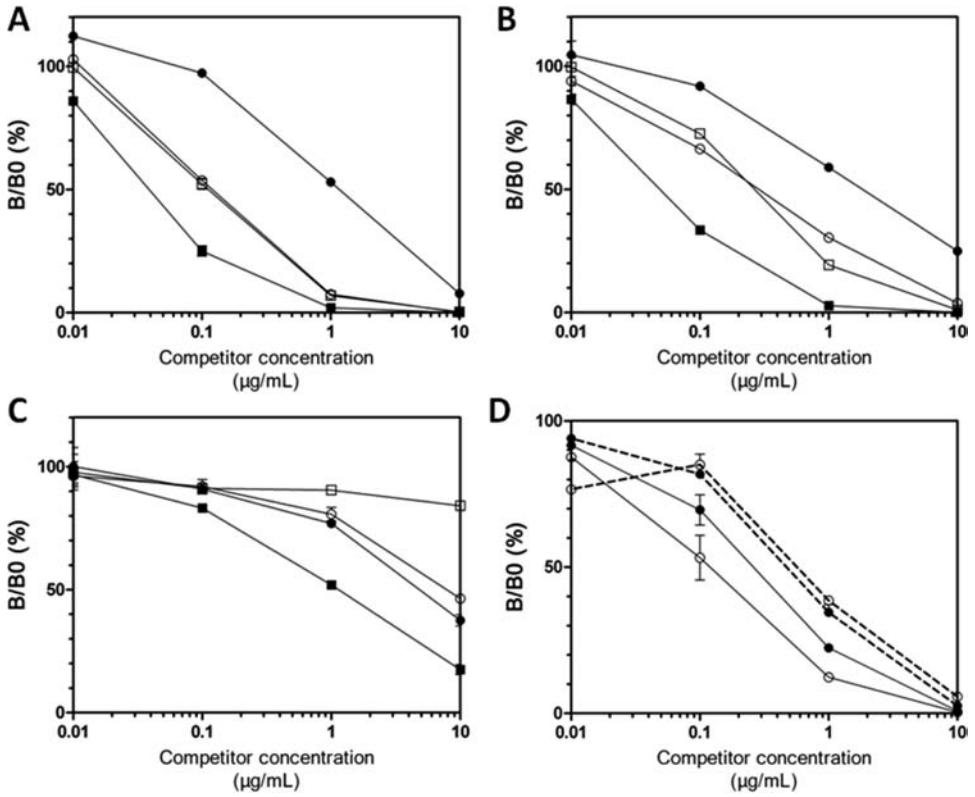

Figure 3. Immunoreactivity of BLG-derived products. Inhibition of the binding of labeled BLG to mAbs specific of BLG region (41−60) (A), (133−143) (B), or (148−162) (C) by nBLG (●), dBLG (■), CNBr-nBLG (○), or CNBr-dBLG (□). (D) Inhibition of the binding to mAb specific of BLG region (41−60) (solid line) or (74−84) (dotted line) by CNBr-dBLG (●) or synthetic peptide (25−107) (○).

very low, probably combining low mAb affinity and/or chemical modification effects, that is, both reduction/carbomethylation and CNBr hydrolysis. Interestingly, the immunoreactivities of nBLG and CNBr-nBLG were comparable, further suggesting that this sequence is mainly present in the MW 15748 entity. Finally, the binding capacity of synthetic peptide (25−107) to mAb directed against peptides (74−84) and (41−60) was comparable to that of the corresponding fragment in the CNBr-dBLG hydrolysate (Figure 3D). Altogether, these results demonstrated that hydrolysates and synthetic peptides retained most of the immunoreactivity of BLG.

Administration of BLG-Derived Products to Mice and Effect on a Further Sensitization. We have recently demonstrated the efficiency of tolerance induction after gavage with native BLG but not with BLG after trypsin hydrolysis.[9] The effect of chemical denaturation (to break S−S bridges) and partial hydrolysis of BLG on specific oral tolerance induced by gavage was then further assessed by administering nBLG, dBLG, or their CNBr hydrolysates.

After sensitization, mice previously gavaged with PBS presented a clear Th2 profile with high levels of BLG-specific IgE (Figure 4A) and IgG1 antibodies (Figure 4B) and intense secretion of IL-4, IL-5, IL-10, GM-CSF, and IL-13 with low secretion of Th1 (IFNγ) and Th17 (IL-17) related cytokines (Figure 4C). BLG-specific IgE and IgG1 antibody productions were significantly reduced by a prior ig administration of nBLG, dBLG, and whole CNBr hydrolysate of nBLG (Figure 4A,B; $p < 0.05$, Kruskall−Wallis and Dunn's multiple-comparison post-test when compared to PBS group). Conversely, gavage with whole CNBr hydrolysate of dBLG led to levels of specific IgE and IgG1 antibodies similar to those observed in the control group gavaged with PBS. However, a significant difference between CNBr-dBLG and PBS mice was observed when using the t test ($p = 0.001$ and $p = 0.006$ for IgE and IgG1, respectively; unpaired t test with Welch's correction). Using the same t test, nBLG- and dBLG-treated mice demonstrated significantly reduced IgE ($p = 0.02$) and IgG1 ($p = 0.01$ and $p = 0.001$, respectively) when compared to CNBr-dBLG treated mice. BLG-specific cytokine secretions were highly reduced in all groups of mice treated with BLG-derived products when compared to PBS group (Figure 4C). IL-5 secretions even correlated with IgE and IgG1 concentrations. However, it is worth noting that a total inhibition of both Th2, Th1, and Th17 cytokines is observed only in the nBLG-gavaged group.

To further study the immunomodulatory potency of BLG-derived peptides, an additional experiment was conducted using synthetic peptides. Peptide (25−107), corresponding to the largest and main fragment generated after CNBr hydrolysis and representing 50% of the BLG sequence, and a smaller one, (84−106), which had been suggested to be tolerogenic in a previous study,[35] were then ig administered to mice at an equivalent molar quantity to that contained in nBLG used as a positive control of induced tolerance. Mice were then sensitized as previously described. For both peptides, no immunomodulatory potential was observed because BLG-specific IgE and IgG1 antibody productions were comparable to those observed in control (PBS) mice using nonparametric Kruskall−Wallis and Dunn's multiple-comparison post test (Figure 5A,B), whereas the efficiency of nBLG to induce oral tolerance was once again evidenced. Statistical analysis using an unpaired t test with Welch's correction revealed a significant difference for

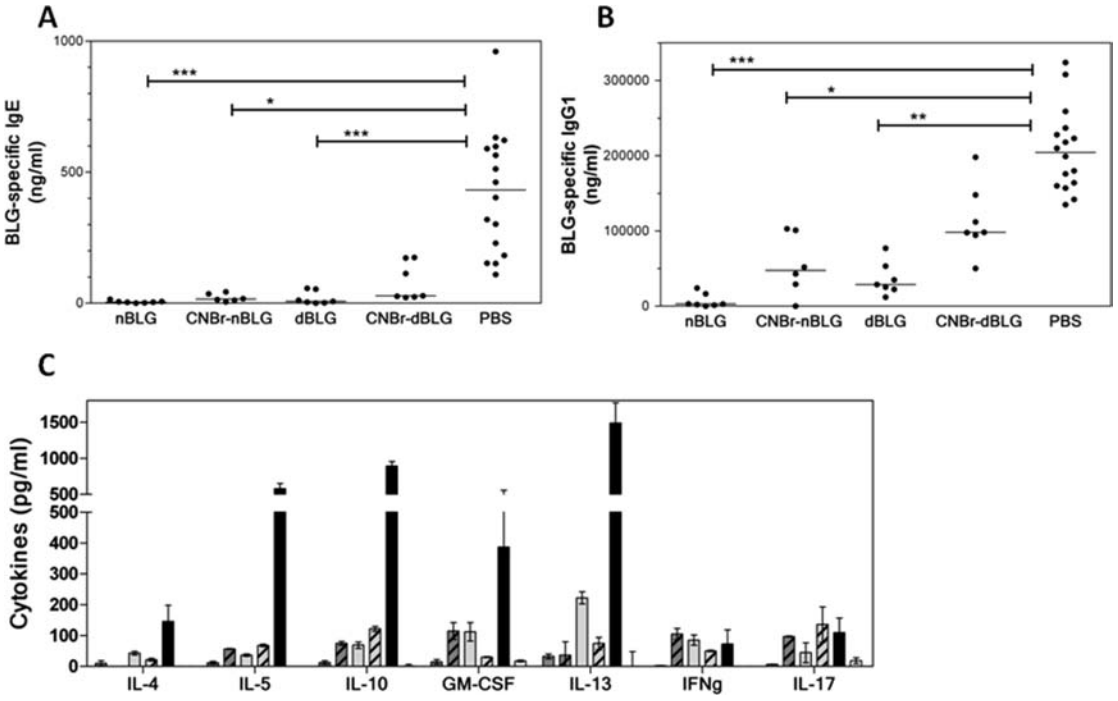

Figure 4. BLG-specific humoral and cellular immune responses of mice gavaged with nBLG, dBLG, or corresponding whole CNBr hydrolysates and then experimentally sensitized. Mice were gavaged with BLG-derived products or PBS as a control and then experimentally sensitized with nBLG. BLG-specific IgE (A) and IgG1 (B) antibodies were quantified on individual sera collected on day 33. Statistical significance between specified groups using Kruskall–Wallis and Dunn's multiple-comparison post-test is indicated (*, $p < 0.05$; **, $0.01 < p < 0.001$; ***, $p < 0.001$). The same results were obtained for specific IgG2a (not shown). (C) BLG-specific cytokine secretion was measured on supernatant of ex vivo restimulated splenocytes using Luminex technology. BLG-specific secretion was obtained after subtraction of nonspecific secretion induced by ovalbumin. No statistical analysis was performed because results are expressed as means of duplicate determinations on supernatants from spleens pooled within pretreatment groups. Mice were gavaged with PBS (black bars), nBLG (dark gray bars), CNBr-nBLG (dark gray slashed bars), dBLG (light gray bars), or CNBR-dBLG (light gray slashed bars); naïve mice (white bars).

BLG-specific IgE antibody concentrations between mice receiving peptide (25–107) and PBS group ($p = 0.04$), which was not further evidenced for IgG1 antibodies. Using the same test, BLG-specific IgE antibody concentration was significantly reduced in the nBLG group when compared to the (25–107) group ($p = 0.025$). Cytokine secretion by BLG-reactivated splenocytes from peptide-treated mice was comparable to that observed for PBS mice (Figure 5C). Altogether, these results demonstrated that none of the peptides tested individually is immunomodulatory in this model.

Splenocytes isolated from mice gavaged with PBS or with synthetic peptide (25–107) or (84–106) and then sensitized to BLG, that is, demonstrating efficient sensitization, were also restimulated ex vivo with the synthetic peptides. As previously shown, secretion of Th2 cytokines was always observed after ex vivo reactivation with BLG, but no cytokine secretion was observed when using peptide (84–106) for reactivation, as shown for IL-13 in Figure 5D, then suggesting this peptide does not contain T-cell epitopes. Conversely, peptide (25–107) induced a specific ex vivo cytokine secretion.

Interestingly, no restricted tolerance to BLG peptide (25–107) was induced in the peptide-treated groups, as shown by the IgE and IgG1 antibody responses against peptide (25–107) that were not different when peptide-treated mice were compared to PBS control mice (Figure 6). Conversely, the same results as shown in Figure 4A,B, that is, decreased antibody concentrations, were obtained when sera from nBLG, dBLG, and CNBr hydrolysate-treated mice were analyzed on BLG peptide (25–107) coated plates (data not shown).

DISCUSSION

In the present study, we have analyzed the immunomodulatory potential on a further allergic sensitization of BLG, either as a native protein or after chemical denaturation, and of the corresponding CNBr hydrolysates. Two relevant synthetic peptides were also assessed.

CNBr hydrolysates were first finely characterized. As a contamination as low as 1.25% of undegraded BLG in the CNBr fractions, corresponding to 0.05 mg of BLG, would have led to partial tolerance irrespective of the effect of the peptides,[9] we first ascertained that no intact BLG was present in these fractions. CNBr hydrolysates were then analyzed by RP-HPLC and mass spectrometry, allowing identification of expected peptides but also observation of incomplete hydrolysis of bonds such as Met^{107}–Glu^{108} and Met^{145}–His^{146}. Electrophoresis and mass spectrometry analysis also evidenced the presence of disulfide bridges in the fragments produced by the CNBr hydrolysis of native BLG. Finally, the immunoreactivity of the fragments was analyzed by competitive enzyme immunoassays using anti-BLG mouse monoclonal antibodies of well-characterized epitope specificity. Although some B-cell epitopes were not fully accessible in the native protein, CNBr hydrolysates of nBLG and dBLG demonstrated the same immunoreactivity (i.e., binding capacity) for mAb specific to regions (41–60) and (133–143) of BLG. Altogether, these results demonstrated that the hydrolysis products correspond to a pool of peptides of various sizes due to partial hydrolysis that can be still associated by S–S bridges and that retain the immunoreactivity of BLG. Binding studies using mAb directed

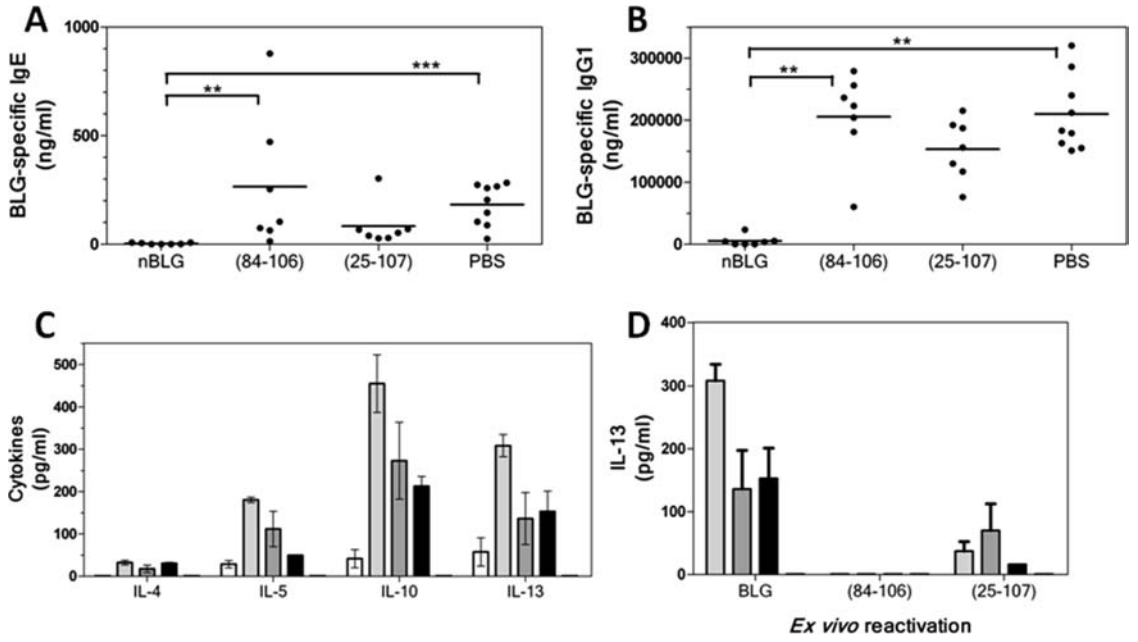

Figure 5. BLG-specific humoral and cellular immune responses in mice gavaged with nBLG or synthetic peptides and then experimentally sensitized. Mice were gavaged with nBLG, synthetic peptides, or PBS as a control and then experimentally sensitized with nBLG. BLG-specific IgE (A) and IgG1 (B) antibodies were quantified on individual sera collected on day 30. Statistical significance between specified groups using Kruskall–Wallis and Dunn's multiple-comparison post-test are indicated (*, $p < 0.05$; **, $0.01 < p < 0.001$; ***, $p < 0.001$). (C) Cytokine secretion by BLG-reactivated pooled spleen cells in mice gavaged with nBLG (white bars), synthetic peptide (84–106) (light gray bars), synthetic peptide (25–107) (dark gray bars), or PBS (black bars). Naïve mice are also shown (hatched bars, no specific secretion detected). (D) IL-13 secreted by splenocytes reactivated by BLG, synthetic peptide (84–106), or synthetic peptide (25–107) (*x*-axis legend). Splenocytes were isolated from mice gavaged with synthetic peptides (84–106) (light gray bars), synthetic peptide (25–107) (dark gray bars), or PBS (black bars). Naíve mice are also shown (hatched bars, no specific secretion detected). In no case was secretion detected in BLG-tolerized mice (not shown).

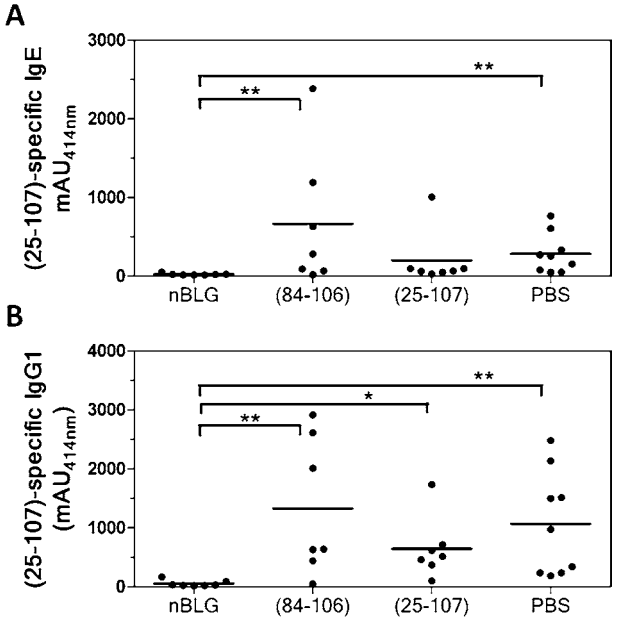

Figure 6. IgE (A) and IgG1 (B) specific antibodies to synthetic peptide (25–107) in mice gavaged with nBLG or synthetic peptides and then experimentally sensitized. Mice were gavaged with nBLG or synthetic peptides and then experimentally sensitized with nBLG. Peptide (25–107)-specific IgE and IgG1 antibodies were quantified on plates coated with the synthetic peptide using individual sera collected on day 30 and diluted 1/50 and 1/5000, respectively. Statistical significance between specified groups using Kruskall–Wallis and Dunn's multiple-comparison post-test are indicated (*, $p < 0.05$; **, $0.01 < p < 0.001$; ***, $p < 0.001$).

against peptide (74–84) and (41–60) also demonstrated that the immunoreactivity of synthetic peptide (25–107) was equivalent to that of the corresponding fragment present in CNBr-dBLG hydrolysate.

Effect of gavage with nBLG, dBLG, and their CNBr hydrolysates on a further experimental sensitization to BLG was then assessed in mice. As previously observed with this experimental model,[9] gavage with native BLG induces an efficient tolerance preventing further induction of BLG-specific IgE and IgG1 and Th2-cytokine secretion by BLG-reactivated splenocytes after experimental sensitization. Associated Th1 and Th17 cytokines were also significantly inhibited. Chemical denaturation of BLG slightly reduced its tolerogenic potential in this model as dBLG was statistically as efficient as nBLG for inhibiting further IgE and IgG1 specific responses, but cytokine secretion was not totally abolished. The same results were observed using nBLG after CNBr hydrolysis, which is likely to result from the presence of an association of peptides linked by S–S bridges of 15748 kDa apparent MW, corresponding to 86% of the BLG MW in this hydrolysate. The immunomodulatory potency of CNBr hydrolysate of dBLG was far less obvious at the humoral level. When all groups in the statistical analysis were considered, BLG-specific IgE and IgG1 antibody concentrations were not significantly different from those observed in PBS control mice. However, Th2 cytokine secretion was also reduced in mice administered CNBr-dBLG, suggesting that those hydrolysates are more potent in preventing further sensitization compared to trypsin hydrolysates that did not allow inhibition of Th2 cytokine secretions at all[9] (Adel-Patient, unpublished results). We then assessed the immunomodulatory potential of synthetic peptide (25–107),

which represents 50% of the BLG sequence and is a main component of CNBr hydrolysates. Surprisingly, it did not decrease further sensitization, either at the cellular or at the humoral level. Accordingly, a smaller BLG-derived peptide, that is, (84−106), was inefficient. Several studies have been conducted in BALB/c mice to determine T-cell epitopes of BLG, using either synthetic or tryptic and chymotryptic peptides. According to these studies, BLG region (25−107) contains several identified major T-cell epitopes, such as sequences (62−76) and (42−56),[36] (41−60) and (59−82),[37] and (60−85) and (73−94).[38] This was also confirmed in the present study as synthetic peptide (25−107) was demonstrated to induce Th2 cytokine production by splenocytes from BLG-sensitized mice (Figure 5D). The synthetic peptide (84−106) does not induce significant cytokine production, which further suggests the absence of T-cell epitopes identified in this sequence and may explain its total inefficiency in the present study.

Altogether, these results demonstrate that, in the BALB/c mouse model, partially hydrolyzed BLG derived from intact BLG can prevent further sensitization, which is more efficient when disulfide bridges are maintained between the peptides generated by the hydrolysis. This is not the case when nBLG extensively hydrolyzed by trypsin[9] or an isolated synthetic peptide even as large as sequence (25−107) is used. This thus suggests that the immunomodulatory potential is mainly supported by the combination/association of the generated peptides, including large ones resulting from partial hydrolysis. Such complex compounds may be present in commercial partially hydrolyzed whey formulas currently used as dietary products, as well as possible low levels of intact native or denatured protein, and both may account for the observed tolerogenic potential of such formulas. Low-dose exposure to these entities would then favor the induction of tolerance via the efficient induction of Treg[9,20] and limit the risk of sensitization.[16,17,20] Further studies of the antigen processing by dendritic cells of the gut-associated lymphoid tissue, notably by $CD103^+$ dendritic cells, and of the potency to locally and systemically induce Treg would then be of interest to elucidate the mechanisms involved in the immunomodulatory properties of the identified entities, that is, peptide associations/aggregates or whole protein, and substantiate their use in milk formulas for the prevention or treatment of allergy to cow's milk proteins.

AUTHOR INFORMATION

Corresponding Author

*Postal address: Unité INRA d'Immuno-Allergie Alimentaire; IBiTec-S/SPI − Bat 136, CEA de Saclay, 91191 Gif-sur-Yvette cedex, France. E-mail: karine.adel-patient@cea.fr Fax: (33) 1 69085907. Phone: (33) 1 69089225.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

BLG, bovine β-lactoglobulin; CNBr, cyanogen bromide; dBLG, chemically denatured BLG; LP, lamina propria; MLN, mesenteric lymph nodes; nBLG, native BLG; OT, oral tolerance; Treg, regulatory T cells.

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