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Specific effects of denaturation, hydrolysis and exposure to *Lactococcus lactis* on bovine β -lactoglobulin transepithelial transport, antigenicity and allergenicity

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Clinical and Experimental Allergy

Summary

Background Food allergy in developed countries represents a growing concern as reflected by epidemiological studies, indicating that up to 4% of the overall population is affected. Reduction of symptoms takes place following evicton or processing of some allergens. However, it cannot be predicted which structural changes will be associated with significant effects on the allergenicity.

Objective To determine how various treatments of bovine β -lactoglobulin (BLG) used as a model antigen alters its immunoreactivity and transepithelial transport, and whether this correlates with reduced allergenicity using an *in vitro* basophil activation assay.

Methods BLG was subjected to reduction/alkylation, trypsin digestion or exposed to *Lactococcus lactis*. The remaining immunoreactivity toward IgG raised against native BLG was assessed by ELISA. Transepithelial transport of BLG and derivatives was examined using polarized Caco-2 cell monolayers mimicking the intestinal epithelium. Selective passage of tryptic peptides was determined using colchicine and cytochalasin D. Basophil activation was measured following stimulation with BLG and derivatives.

Results Reduction/alkylation, trypsin digestion or incubation with *L. lactis* was associated with decreased BLG recognition by IgG antibodies raised against the native protein. All treatments also resulted in a more efficient transepithelial transport of BLG. BLG crossed the Caco-2 monolayer through passage across the cell, whereas tryptic peptides followed both the para- and transcellular routes. With the exception of denaturation by reduction/alkylation, cross-linking of IgE antibodies by BLG derivatives led to lower basophil degranulation.

Conclusion *In vitro* dissection of antigenicity and allergenicity may be a valid and convenient alternative to evaluate the effects of biotechnological processing on dietary proteins. In addition, it can help to define the molecular and cellular mechanisms that will provide improved means of diagnosis and possibly therapy of food-allergic disorders.

Keywords allergenicity, basophil activation, immunoreactivity, intestinal epithelial permeability, lactic acid bacteria, protein processing

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Introduction

The mucosal immune response to dietary proteins is dependent upon the gastrointestinal luminal denaturation and enzymatic hydrolysis of antigenic determinants [1, 2]. Different factors such as bile salts and low pH, as well as tryptic and peptic digestion have been shown to reduce food protein immunogenicity [3, 4]. In the intestinal microbiota, lactic acid bacteria were also reported to

contribute to luminal processing and thereby to give rise to peptides with immunomodulatory properties [5, 6]. The maintenance of a non-inflammatory response to food antigens is also achieved under non-pathophysiological conditions by the single layer of intestinal epithelial cells functioning in nutrient absorption/processing and as a barrier with selective permeability [7].

Abnormal intestinal permeability is a common mark of food hypersensitivity disorders [8]. This is accompanied

by increased transport of intact food proteins across the gut epithelial barrier, thus contributing to the immunologically mediated pathophysiology of food allergy [9]. In terms of mechanism, this involves cross-linking of IgE bound to the Fcε receptor I on basophils/mast cells by antigen proteins [10, 11]. This induces the secretion of effector/immunoregulatory products, which further enhance epithelial permeability [12], and leads to qualitatively altered transepithelial transport of dietary proteins [13]. Fragmentation or structural alteration of the allergen is thus considered as a valuable means of preventing IgE binding and subsequent deleterious effect associated with mast cell mediator release [14].

Bovine β-lactoglobulin (BLG) is the main proteinaceous component of whey and is considered as a major cow's milk allergen [15]. Similar to a number of other food allergens, it has been documented to display a rather high resistance to proteolytic degradation [16]. Moreover, BLG was shown to be efficiently absorbed by human and rabbit intestinal explants, and to cross the epithelial barrier partly as an intact antigenic protein [17, 18]. There, it can contribute to the onset of the allergic response in an IgE-dependent manner. However, tolerance to BLG can be induced in rat and mouse following oral administration of cow's milk formulae based on intact whey proteins or a tryptic hydrolysate thereof [19, 20].

We set up two *in vitro* assays based on either polarized Caco-2 intestinal epithelial cells or rat basophilic leukaemia RBL-2H3 cells, a tumour analogue of rat mucosal mast cells, to examine how BLG denaturation, enzymatic hydrolysis or incubation with *Lactococcus lactis* affects transepithelial transport and mast cell activation. BLG derivatives display a higher rate of transport across Caco-2 monolayers, as well as lower immunoreactivity. BLG enzymatic processing generates peptides, which differentially cross the intestinal epithelium, both in terms of transport rate and pathway. Tryptic digestion or BLG incubation with *L. lactis* was found to lower BLG allergenicity significantly as assessed by degranulation of RBL-2H3 cells sensitized with BLG-specific IgE. In contrast, BLG denaturation did not result in any detectable reduction of allergenicity. Hence, the *in vitro* models described in this work allow identification of subtle differences in the antigenic and allergenic properties of an allergen protein model.

Materials and methods

Preparation of biotinylated β-lactoglobulin

Batches of 10 mg of 3 × crystallized BLG (Sigma, St. Louis, MO, USA) dissolved in PBS were treated with 1 mg of sulphy-NHS-LC-biotin (Pierce, Rockford, IL, USA) for 30 min at room temperature under gentle agitation. Unreacted biotin was removed by gel filtration using Sepha-

dex G-25 prepacked PD-10 desalting columns (Amersham Biosciences, Uppsala, Sweden) equilibrated and run with PBS. Fractions of 1 mL were recovered and the presence of biotinylated BLG (BLGbiot) was verified by immunoblot analysis using horseradish peroxidase (HRP)-conjugated Extravidin (Sigma).

Biotinylation of β-lactoglobulin peptides

The amino acid sequences of BLG peptides and control peptide bradykinin are listed in Fig. 4c. Peptides were synthesized using standard solid-phase F-moc chemistry (Applied Biosystems, Foster City, CA, USA). Peptides were purified by reverse-phase HPLC using a Vydac C18 column, and purity was confirmed by mass spectrometry. Reduction of cysteine sulphhydryl groups in peptide (1 mM in PBS) was achieved using 5 mM Tris(2-carboxyethyl) phosphine (Pierce). The excess of reducing agent was removed by passage over a desalting G-10 column (Amersham Biosciences) equilibrated and run with PBS. Biotinylation was achieved upon addition of 2.5 mM PEO-maleimide-biotin (Pierce) and overnight incubation at 4 °C, and the excess of biotinylation reagent was removed as above.

Preparation of reduced and alkylated β-lactoglobulin and biotinylated β-lactoglobulin

1.5 mg of BLG or BLGbiot in PBS was denatured in 85% methanol (final concentration) for 2 h at −20 °C. The protein pellet resulting from centrifugation at 15 000 *g* for 1 h at 4 °C was dried under nitrogen flushing, resuspended in 300 µL of 100 mM dithiothreitol and incubated for 25 min at room temperature. Following this initial reduction, the volume was brought to 800 µL with water, 200 µL of 1 M N-ethylmaleimide (NEM) in ethanol was added and alkylation was carried out for 20 min at room temperature. After addition of 1.5 mL of 100 mM sodium bicarbonate (pH 8.3), the protein was separated from the excess of reagents by gel filtration using a prepacked PD-10 desalting column in 100 mM sodium bicarbonate (pH 8.3). The eluate was quick-frozen on dry ice before lyophilization.

Preparation of total tryptic hydrolysates of β-lactoglobulin and biotinylated β-lactoglobulin

1 mg of BLG or BLGbiot diluted at 0.05% (w/v) in PBS containing 10 mM CaCl₂ was digested overnight at 40 °C upon incubation with 1 mg of TPCK-treated trypsin from bovine pancreas (7500 U/mg; Fluka, Buchs, Switzerland). The reaction was stopped by inactivation of the enzyme at 85 °C for 5 min.

Bacterial strains and growth conditions

The PrtP[−] PrtM[−] Lac[−] *L. lactis* strain MG1363 (referred to as wtLl in the paper) [21], which lacks PrtP extracellular

proteinase activity, was transformed with an expression vector encoding the full-length PrtB proteinase of *L. bulgaricus* [22], yielding recLl. Bacteria were grown at 30 °C in M17 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 1% glucose (GM17), and chloramphenicol at 10 µg/mL was added when required. Stationary-phase cultures reached approximately 3 OD₆₀₀ units, one unit corresponding to 3.5×10^8 colony-forming units (CFU) per mL.

Lactococcus lactis-mediated processing studies

Bacteria (wtLl and recLl) of stationary-phase cultures were recovered by centrifugation (2000 g, 5 min), washed in PBS and resuspended at 2×10^8 CFU/mL in fresh GM17 medium. One-milliliter cultures in 1.5 mL reaction tubes were incubated with 10 µg of either BLG or BLGbiot for 60 min at 30 °C. Bacteria were removed by centrifugation (4000 g for 5 min) and the protein in culture supernatant was analysed by ELISA as described below. Alternatively, 10 µL of culture supernatant was subjected to SDS-PAGE and analysed by immunoblotting, as described below.

Direct enzyme-linked immunosorbent assay for detection of biotinylated β-lactoglobulin and derivatives

The direct assay allows detection of the protein irrespective of its conformation and integrity. Serial dilutions of BLGbiot-containing samples (100 µL) were coated overnight at 4 °C into 96-well MaxiSorp ELISA plates (Nunc, Roskilde, Denmark). Wells were blocked for 45 min at 37 °C with PBS containing 0.05% Tween-20 (PBS/T) and 1% fish gelatin. After three washes in PBS/T, detection was performed using 100 µL of HRP-conjugated Extravidin (1 : 800; Sigma) in PBS/T for 45 min at 37 °C. After three additional washes in PBS/T, the reactions were developed with 100 µL of 1,2-phenylenediamine as a chromogen, and stopped with 100 µL of 2 M H₂SO₄. Absorbance was measured at 490 nm, with 620 nm as the reference wavelength.

Direct enzyme-linked immunosorbent assay for detection of β-lactoglobulin and derivatives

BLG and derivatives were detected as described above for biotinylated products, using 100 µL of biotinylated rabbit anti-BLG IgG (1 : 2000; R. Fritsché, unpublished data) diluted in PBS/T as a primary reagent. All other steps were identical to those for direct ELISA specific for BLGbiot.

Indirect enzyme-linked immunosorbent assay

The indirect assay reveals possible conformational differences between protein samples resulting from transport or processing by bacteria. The wells of MaxiSorp ELISA

plates were coated overnight at 4 °C with 100 µL of rabbit anti-BLG antiserum (1 : 250) used as capture reagent, and prepared as described previously [22]. Subsequent blocking, detection and developing steps were performed as described above for the direct ELISA.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblot analysis

Samples were mixed with $2 \times$ non reducing gel loading buffer (100 mM Tris, 4% SDS, 0.2% bromophenol blue, 20% glycerol, pH 6.8), boiled for 3 min, separated on SDS-15% polyacrylamide gels and transferred onto PVDF membranes (Millipore). Membranes were blocked with 1% fish gelatin in PBS/T, washed and probed with either rabbit anti-BLG antiserum (1 : 10 000), followed by HRP-conjugated goat anti-rabbit immunoglobulin G (1 : 3000; BD Biosciences, San Diego, CA, USA), or directly with HRP-conjugated Extravidin (1 : 10 000) diluted in PBS/T. Incubation time was 1 h at room temperature. After washing (2×5 min and 2×10 min washes) in PBS/T, bound reagents were detected using the UptiLight HRP blot chemiluminescent substrate according to the manufacturer's instructions (Interchim, Montluçon, France).

Caco-2 cell culture

The Caco-2 cell line, derived from a human colorectal carcinoma, was obtained from the American Type Culture Collection (Rockville, MD, USA), and was used between passage numbers 30 and 40. The cells were grown and seeded onto 1.0 cm² Snapwells (0.4-µm pore size; Corning, Acton, MA, USA) as published [23]. The medium was changed every 2 days and the monolayers used from day 21 after seeding. The integrity of the Caco-2 cell monolayers was checked by measuring the transepithelial electrical resistance (TER), using a Millicell-ERS device (Millipore, Bedford, MA, USA). TER values of well-differentiated cell monolayers, blanked for values obtained with filters alone, were in the range of 300–500 Ω cm².

Transport studies

Caco-2 cell monolayers were washed twice with HBSS (Sigma); in some sets of experiments, cells were preincubated for 45 min at 37 °C with either 2 µM colchicine (Fluka) or 0.2 µM cytochalasin D (Fluka) in HBSS, corresponding to concentrations optimal to assay transcytotic and paracellular transport without debilitating TER. 25 µg of BLG, 20 µg of BLGbiot, or equimolar amounts of reduced and alkylated BLGbiot (rNEM:BLGbiot), total tryptic hydrolysates of BLGbiot (TTH:BLGbiot) or biotinylated peptide samples were applied to the apical chamber in a final volume of 500 µL of HBSS. Following incubation at 37 °C for 60 min, the content from both the apical and

basolateral compartments was assayed for HRP-conjugated Extravidin-binding using direct ELISA.

Rat sensitization to β -lactoglobulin

For the preparation of anti-BLG IgE-containing sera, Sprague–Dawley rats (Charles River Laboratories, Les Oncins, France), maintained on a milk-free diet, were immunized intraperitoneally with 100 μ g of BLG mixed with 0.5 mL of 3% alum (Sigma) in PBS. Booster immunizations were carried out on days 14 and 28, with 10 μ g of BLG mixed with 0.5 mL of 3% alum. Blood was obtained by cardiac puncture under isoflurane anaesthesia on day 35, and seric total IgE was determined by ELISA, as described previously [24].

RBL-2H3 cell culture and degranulation assay

RBL-2H3 cells, a kind gift from Dr U. Wiedermann (Medical University of Vienna, Austria), were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FCS, 2 mM L-glutamine (Sigma), 2 mM sodium pyruvate (Sigma), 10 mM HEPES, 0.1 mM 2-mercaptoethanol, 100 U/mL penicillin and 100 μ g/mL streptomycin. For degranulation assays, cells were detached using 0.25% trypsin and 0.5 mM EDTA, seeded in the wells of 96-well tissue culture plates (4×10^4 cells per well) and incubated for 20 h at 37 °C. Cells were sensitized for 2 h at 37 °C with appropriate dilutions of rat anti-BLG IgE-containing serum in RPMI 1640 medium. IgE cross-linking was performed using 100 μ L of serial dilutions of BLG or derivatives for 30 min at 37 °C. The supernatants were cleared by centrifugation, and the release of β -hexosaminidase was determined as described [25].

Fluorescence microscopy

Caco-2 cell monolayers were washed and pulsed for the times indicated with 25 μ g of BLG in HBSS at 37 °C. After two washes in HBSS, cells were fixed with methanol:acetic acid (3 : 1) at –20 °C for 5 min. After washing with PBS/T, non-specific binding sites were blocked with PBS/T containing 1% fish gelatin (Sigma) for 60 min at room temperature. The cells were subsequently incubated for 60 min at room temperature with rabbit anti-BLG antiserum (1 : 500 dilution in PBS/T). After three washes in PBS/T, cells were incubated for 45 min at room temperature with 1 : 50 fluorescein-conjugated swine anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) in PBS/T. After three further washes in PBS/T, filters were cut out of their holders, placed on slides, air dried, mounted using Slow-Fade Light Antifade Kit (Molecular Probes, Eugene, OR, USA), coverslipped and sealed. Observations were performed using an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany) coupled to a colour Cool-view camera (Photonic Science, East Sussex, UK).

Laser scanning confocal microscopy

Caco-2 cell monolayers were pulsed with 20 μ g of BLGbiot, and processed as above for fluorescence microscopy, using fluorescein-conjugated streptavidin (Dako, 1 : 100 dilution) in PBST for detection. Observations were performed using a laser scanning confocal microscope (Zeiss LSM 510 Meta; Zeiss) equipped with an argon-krypton laser. All images were identically processed using the LSM 5 Image Browser application (Zeiss).

Statistical analysis

Standard deviations were calculated using the function STDEVP from the Excel 98 application for Apple Macintosh (Cupertino, CA, USA). Statistical significance was determined using the two-tailed non-parametric Mann–Whitney test. *P* values were calculated using InStat 2.01 application (GraphPad, San Diego, CA, USA).

Results

Comparative immunoreactivity of β -lactoglobulin, biotinylated β -lactoglobulin and derivatives

As a substitute to denaturation and proteolysis taking place during gastrointestinal transit, we prepared BLG derivatives by reduction/alkylation (reduced and alkylated BLG (rNEM:BLG)) and tryptic digestion (total tryptic hydrolysates of BLG (TTH:BLG)). When analysed by non-reducing SDS-PAGE and immunoblot, these derivatives were found to exhibit different migration patterns, indicative of the efficacy of both treatments (Fig. 1a). The absence of a signal in the TTH:BLG lane showed that tryptic digestion abolished recognition by a BLG-specific antiserum under denaturing conditions. Under non-denaturing conditions, the immunoreactivity of rNEM:BLG and TTH:BLG towards the BLG-specific antiserum in the indirect ELISA assay decreased drastically ($72 \pm 2.5\%$ and $99 \pm 1\%$, respectively) as compared with BLG (Fig. 1b). Thus, both conformational changes and extensive hydrolysis contribute to destruction of epitopes present in native BLG.

To guarantee detection of the various molecular and structural forms of the protein using HRP-conjugated Extravidin, biotinylated BLG and derivatives (rNEM:BLGbiot and TTH:BLGbiot) were used (Fig. 1c). Concentrations of BLGbiot and derivatives were adjusted on the basis of the signals obtained using direct ELISA (data not shown). Similar migration patterns under non-reducing conditions (Fig. 1c) and immunoreactivity in the indirect ELISA (Fig. 1d) were obtained in comparison with native BLG ($85 \pm 1.5\%$ and $98 \pm 0.9\%$, respectively). In addition, TTH:BLGbiot can be detected, thus allowing to track all forms of BLG in the *in vitro* systems described below.

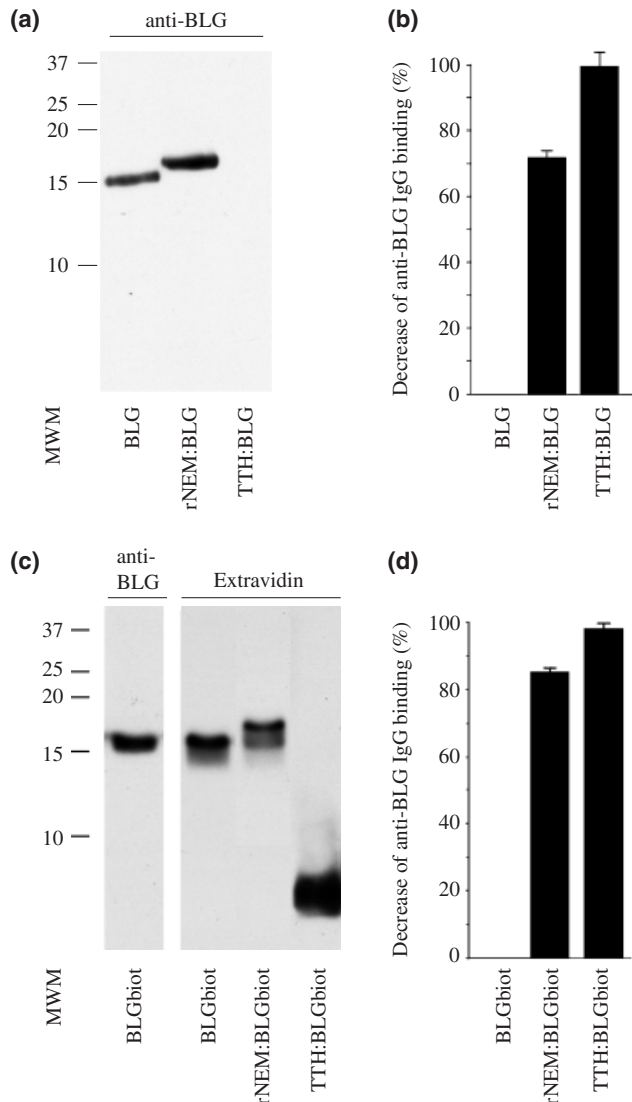


Fig. 1. Immunoreactivity of β -lactoglobulin (BLG), biotinylated BLG and derivatives. (a, c) Immunoblot analysis after separation using Polyacrylamide gel electrophoresis under nonreducing conditions. MWM: molecular weight markers. (b, d) Indirect ELISA using anti-BLG serum for capture. BLG was detected with biotinylated anti-BLG IgG, followed by horseradish peroxidase (HRP)-conjugated Extravidin, and BLGbiot with HRP-conjugated Extravidin. Assessment of changes in immunoreactivity was calculated as described in Materials and methods, and data represent means and standard deviations from three independent experiments.

β -Lactoglobulin alteration by *Lactococcus lactis*

We next assessed the impact of wtLl on the recognition of either BLG or BLGbiot by a specific antiserum. In parallel, we examined the effect of recLl strain designed to express the full-length PrtB proteinase of *Lactobacillus bulgaricus* known to hydrolyze milk caseins with high efficiency [26]. Using indirect ELISA, we found that wtLl only marginally reduced BLG-specific antibody binding, whereas a significant reduction in immunoreactivity was observed after incubation with the recLl strain

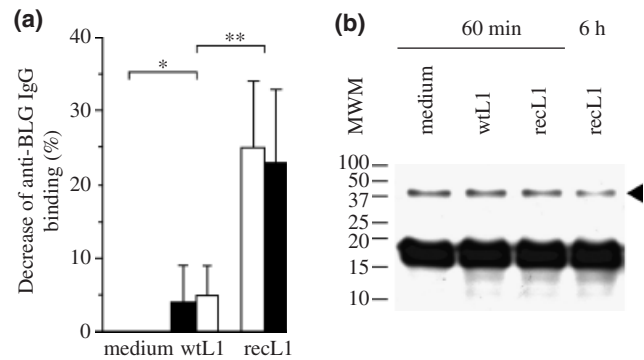


Fig. 2. *Lactococcus lactis*-mediated processing of β -lactoglobulin (BLG) and biotinylated BLG (BLGbiot). (a) Effect of wild-type *L. lactis* (wtLl) or recombinant *L. lactis* (recLl) on the immunoreactivity of BLG (empty bars) and BLGbiot (filled bars). Cell culture supernatants recovered after a 60 min incubation at 30 °C were analysed by indirect enzyme-linked immunosorbent assay using anti-BLG antiserum for capture. Decreased reactivity was measured as indicated in Materials and Methods. * $P < 0.01$, ** $P < 0.0001$. (b) Immunoblot analysis of BLGbiot after incubation for the indicated time with medium as control, wtLl, or recLl. Detection was obtained using Horseradish peroxidase-conjugated Extravidin. The arrowhead marks the migration position of BLG dimers. MWM: molecular weight markers.

($25.4 \pm 9.2\%$ for BLG, and $22.8 \pm 10.9\%$ for BLGbiot, respectively; Fig. 2a).

To ensure that incubation of BLGbiot with *L. lactis* strains did not result in the mere loss of protein sticking on the surface of bacteria, the same mixtures were analysed using direct ELISA. Incubation of BLGbiot with either wtLl or recLl did not alter the protein reactivity to Extravidin as compared with plain medium ($98.0 \pm 7.0\%$ for wtLl, $98.0 \pm 8.2\%$ for recLl strain, as normalized to the signal obtained with plain medium, data not shown). This suggests that the decrease of BLG-specific antiserum binding observed with the PrtB-expressing strain reflects either structural alterations or partial proteolysis catalysed by the protease. To discriminate between these two possibilities, cell culture supernatants containing BLGbiot were analysed by immunoblotting (Fig. 2b). In comparison with BLGbiot kept in plain medium, barely detectable degradation was observed upon incubation with either wtLl or recLl at 60 min and 6 h (Fig. 2b). While confirming the high resistance of BLG to proteolysis [16], this strongly suggests alternative PrtB-catalysed alterations of the protein, for instance deamidation [27].

β -Lactoglobulin transepithelial transport and processing across Caco-2 cell monolayers

We used polarized Caco-2 cell monolayers to evaluate the role of intestinal epithelial cells in the processing of either BLG or BLGbiot. We first established experimental conditions allowing reliable assessment of the release of BLG and BLGbiot into the basolateral compartment. By probing basolateral samples with either BLG-specific

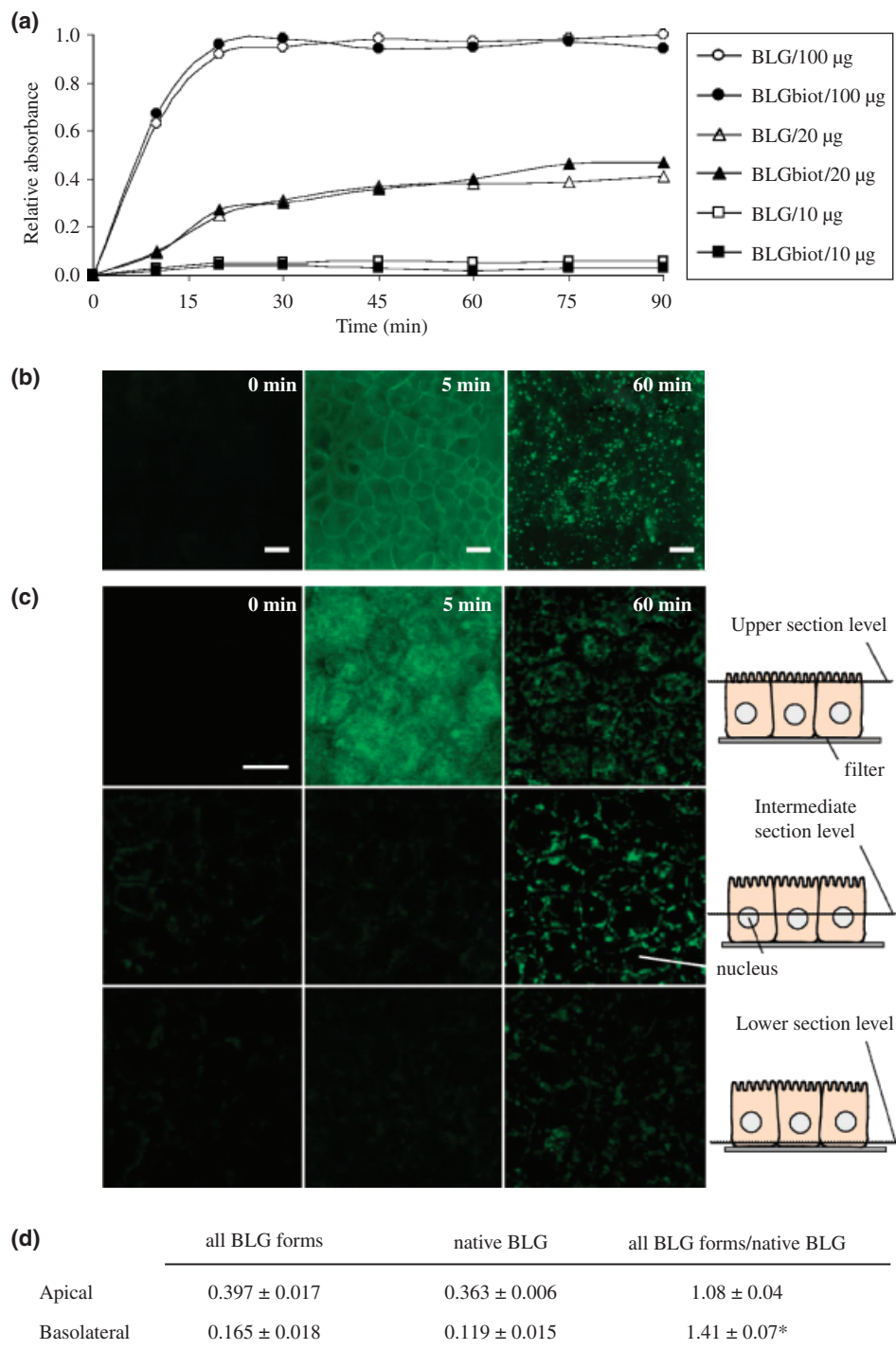


Fig. 3. Transport of β -lactoglobulin (BLG) and biotinylated β -lactoglobulin (BLGbiot) across polarized Caco-2 cell monolayers. (a) Kinetics studies and dose-dependent rate of BLG transport. Caco-2 cells were pulsed with different amounts of BLG or BLGbiot, as indicated, and samples of the basolateral compartment were analysed at different times by direct enzyme-linked immunosorbent assay (ELISA). (b) Kinetics studies of BLG uptake by polarized Caco-2 cells. Caco-2 cell monolayers were pulsed with 25 μ g of BLG for the indicated times, and analysed by fluorescence microscopy using anti-BLG antiserum, followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Bars, 10 μ m. (c) Cellular localization of BLGbiot in polarized Caco-2 cells. Caco-2 cell monolayers were pulsed with 20 μ g of BLGbiot, and analysed by confocal fluorescence microscopy using FITC-conjugated streptavidin. (d) Processing of BLG during passage across polarized Caco-2 cells. Cells were pulsed with 20 μ g of BLGbiot for 60 min, and native or all forms of BLG were analysed by indirect or direct ELISA, respectively. Values are expressed as absorbance units at 490 nm and represent means and standard deviations obtained from four Snapwells of a representative experiment. The highest ratio value for the basolateral compartment indicates BLG processing. $^*P < 0.03$ for the difference between apical and basolateral ratios.

antiserum or HRP-conjugated Extravidin, we found that both BLG and BLGbiot are detected within 10 min after apical delivery, indicating passage across the epithelial cell monolayer with very similar dose-dependent rates (Fig. 3a). Cumulative measurements of the basolateral signal obtained upon apical delivery of 10 µg amounts of protein were below the detection limit. At 100 µg of protein per filter, a plateau was rapidly reached as reported previously [28]. We therefore set as an experimental standard the intermediate protein amount of 20 µg per filter, which generated signals in an appropriate range over a 60 min time frame.

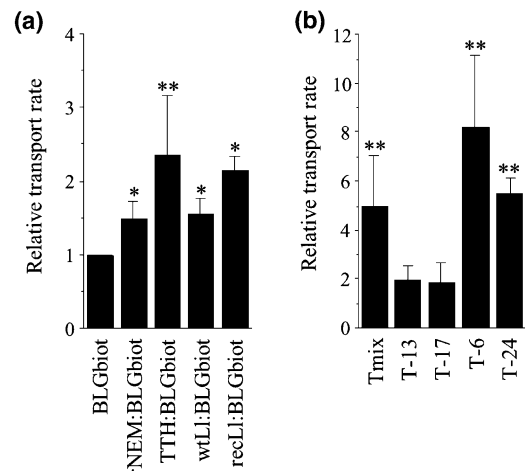
We next verified whether BLG crossed the Caco-2 cell monolayers following the transcytotic route by tracking the protein using fluorescence microscopy (Fig. 3b). While a diffuse pattern of fluorescence compatible with cell surface staining was observed after 5 min of incubation (Fig. 3b, central panel), BLG was detected at 60 min as punctuate structures reminiscent of endocytic vesicles (Fig. 3b, right panel), suggesting a major contribution of the transcytotic pathway. In support of this, the flux of BLG at 60 min was found to be twofold lower after treatment of the Caco-2 cell monolayers with colchicine, an inhibitor of intracellular vesicle transport (data not shown).

Using laser scanning confocal microscopy, we observed BLG predominantly in the apical cell region at 5 min (Fig. 3c, central panels). Moreover, an absence of signal in the intercellular space at the upper section level reflected the integrity of the polarized monolayers and further confirmed weak, if not absent, paracellular transepithelial transport. At 60 min, fluorescence was present at each section level observed (Fig. 3c, right panels), with brighter discrete structures in the central portion of the cell, thus confirming accumulation in endocytic vesicles.

We next wanted to assess the degree of BLGbiot alterations, i.e. denaturation and/or proteolysis, taking place during transcytosis. To this end, we compared the signals obtained by two independent ELISA measurements of either all forms of BLG (direct assay), or native BLG only (indirect assay). The direct assay to indirect assay signal ratio measured at 60 min was found to be significantly higher when calculated from basolateral samples, as compared with apical samples, reflecting a partial loss of BLG structural epitopes during transcytosis of BLGbiot (Fig. 3d). Taken together, immunoreactivity (Figs 1–3) and transport-based data (Fig. 3) indicate that treatments mimicking gastrointestinal luminal and epithelial processing modify BLG structure and hence behaviour in these assays.

Transepithelial transport of biotinylated β-lactoglobulin derivatives across Caco-2 cell monolayers

Next, the rate of transepithelial transport of BLGbiot derivatives (Fig. 1), or BLGbiot exposed to *L. lactis* was



(c) Nomenclature and amino acid sequences of BLG peptides included in Tmix, and bradykinin

^a Numbers in brackets indicate the position of the peptide amino acid residues in the BLG sequence.

^b Residues (one letter IUPAC code) comprised in BLG tryptic peptides and bradykinin are indicated with bold capitals. Extra residues corresponding to original BLG sequences (underlined capitals) or not (plain capitals) were added to either guarantee peptide solubility or to allow cysteine-mediated biotinylation at the N- or C-terminus.

Fig. 4. Transepithelial transport of β-lactoglobulin (BLG) derivatives and peptide fragments. (a) Caco-2 cells were pulsed for 60 min with 20 µg of biotinylated β-lactoglobulin (BLGbiot) or equivalent amounts of BLG derivatives. Samples from the basolateral compartment were analysed by direct enzyme-linked immunosorbent assay. (b) Same set of experiments performed with BLG peptides. Tmix indicates a mixture of four synthetic peptides (T-13, T-6, T-17, T-24) corresponding to BLG tryptic products. In (a) and (b), values are normalized to the signal obtained with BLGbiot, and represent means and standard deviations from at least four experiments. **P* < 0.03, ***P* < 0.01. (c) Sequences of BLG tryptic peptides included in Tmix, and bradykinin used as control.

examined. Caco-2 cells were pulsed for 60 min with either BLGbiot, rNEM:BLGbiot, TTH:BLGbiot or BLGbiot previously incubated with wtLI (wtLI:BLGbiot) or recLI (recLI:BLGbiot). Samples of the basolateral compartment were taken out and analysed by direct ELISA (Fig. 4a). Both rNEM:BLGbiot and TTH:BLGbiot displayed increased rates of transport across the epithelial monolayer (1.4 ± 0.4-fold, and 2.4 ± 0.8-fold, respectively). Similarly, wtLI:BLGbiot and recLI:BLGbiot transepithelial transport

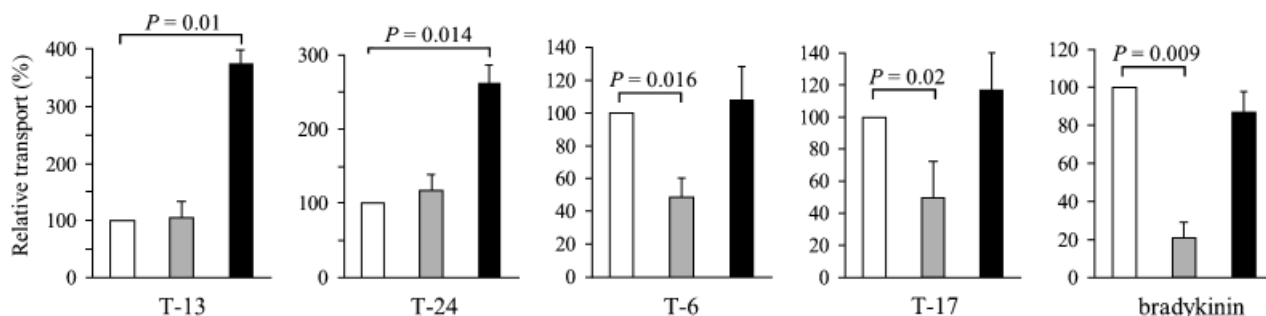


Fig. 5. Mechanistic dissection of the transepithelial transport of β -lactoglobulin tryptic peptides (T-13, T-24, T-6, T-17) and bradykinin. Caco-2 cell monolayers (empty bars), or treated with colchicine (grey bars) or cytochalasin D (filled bars) were pulsed with biotinylated peptides for 60 min. Samples of the basolateral compartment were analysed by enzyme-linked immunosorbent assay using horseradish peroxidase-conjugated Extravidin. For each individual peptide, values are normalized to the signal obtained in the absence of drug (set at 100%), and represent means and standard deviations from at least four experiments.

was found to be more effective (1.6 ± 0.1 -fold and 2.1 ± 0.1 -fold, respectively) as compared with BLGbiot, falling in a range just below that measured with TTH:BLGbiot.

To gain further insight into the transport of TTH:BLGbiot, we used a mix of four synthetic BLG peptides (Tmix), which correspond to tryptic digestion products encompassing residues distributed at the termini or the surface of the protein, and therefore more prone to being soluble (Fig. 4c). To assess their presence in the basolateral compartment, peptides were N- or C-terminally biotinylated via a cysteine residue present in T-24, or added in T-13, T-6 and T-17. As shown in Fig. 4b, Tmix was consistently found to cross the epithelial cell monolayer at a rate higher than BLGbiot (5.0 ± 2.1 fold) or TTH:BLGbiot (2.1 ± 0.9 -fold). This increase in transepithelial transport was shown to be contributed by peptides T-6 (8.2 ± 2.9 fold) and T-24 (6.5 ± 1.3 -fold), which markedly exceeded the transport rate of Tmix, in contrast to T-13 (2.0 ± 0.6 -fold) and T-17 (1.8 ± 0.8 -fold).

We then sought to determine by which cellular pathway BLG-derived peptides were transported across Caco-2 cell monolayers. Pretreatment of epithelial cells with cytochalasin D, a drug that favours the paracellular pathway by opening tight junctions in a dose-dependent manner, resulted in a clear and selective increase of the flux of peptides T-13 and T-24 (Fig. 5). The moderate opening of tight junctions obtained under our experimental conditions (a decrease in TER ranging from 15% to 25% of initial values) was confirmed not to increase the transport of biotinylated bradykinin, a 10-mer peptide used as a control for its documented property to follow the transcytotic route [29]. The transport of peptides T-6 and T-17 was not significantly affected by cytochalasin D treatment either, further confirming the selective mode of action of the drug. Consistent with this, the flux of peptides T-6 and T-17, as well as control peptide bradykinin, was clearly lower after addition of colchicine. This argues for a predominant role of the transcytotic pathway during the

transepithelial transport of these three peptides. Conversely, and consistent with the above data, the flux of peptides T-13 and T-24 was only marginally affected when Caco-2 cells were treated with colchicine. Together, these data reveal that tryptic peptides exhibit the intrinsic property of crossing the epithelial barrier along two different transport pathways with different rates; this accounts for the increased transepithelial transport of TTH:BLGbiot seen previously.

Comparative allergenicity of native vs. processed β -lactoglobulin

Biotechnical processing including heating, proteolysis, and genetic engineering is often seen as a key player in reducing food antigen allergenicity [14]. So far, the present study indicates that destruction of BLG epitopes reflected by differential recognition of BLG derivatives by specific IgG antibodies correlates with increased BLG transport; yet, the relationship with allergenicity is missing. This prompted us to explore the possibility that, in turn, these BLG alterations modulate IgE cross-linking on responding basophils. We first verified the strict IgE-dependency of hexosaminidase release elicited by BLGbiot on RBL-2H3 cells sensitized with BLG-specific IgE (Fig. 6a, filled squares). Subsequent activation experiments were conducted with a concentration of BLGbiot set at 10 ng/mL (Fig. 6a, arrow). We next investigated the hexosaminidase release elicited by the different BLG molecular forms on sensitized RBL-2H3 cells. The triggering effect of rNEM:BLGbiot was found to slightly exceed that of BLGbiot (1.3 ± 0.2 -fold). As expected, stimulation by TTH:BLGbiot resulted in a very significantly reduced degranulation (0.07 ± 0.03 -fold). Noteworthy, the response measured upon stimulation with either wtLI:BLGbiot or recLI:BLGbiot was significantly reduced as compared with BLGbiot as such or left in bacterial GM17 medium alone. We conclude that variations in IgG-mediated immunoreactivity (Figs 1 and 2) do not

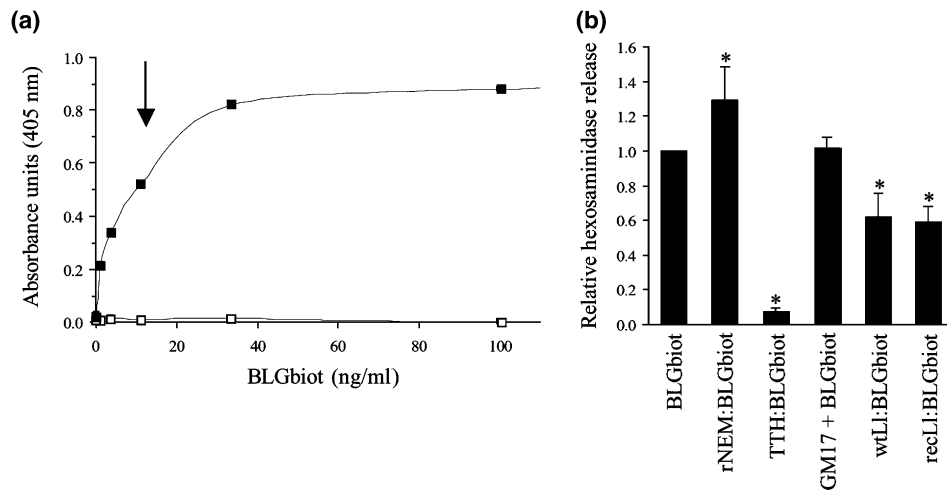


Fig. 6. Hexosaminidase release by RBL-2H3 cells exposed to biotinylated β -lactoglobulin (BLGbiot) and derivatives. (a) Dose-response curve obtained with unsensitized (empty squares) or β -lactoglobulin-specific IgE-sensitized RBL-2H3 cells (filled squares) following stimulation with increasing amounts of BLGbiot. (b) Relative hexosaminidase release measured upon stimulation of sensitized RBL-2H3 cells with 10 ng/mL of BLGbiot or equimolar amounts of derivatives. GM17, medium for bacterial culture. Values are normalized to the signal obtained with BLGbiot, and represent means and standard deviations from three independent experiments. * $P < 0.01$ using BLGbiot as standard.

guarantee the behaviour of the BLG derivatives in terms of IgE cross-linking and ultimately basophil degranulation.

Discussion

The physiologic immune response to dietary proteins is the induction of a state of active unresponsiveness termed oral tolerance, which relies on a controlled processing and transport of antigens that gain access to the mucosal immunocompetent cells [30]. While the identification of the immune players involved has led to significant progresses [31, 32], more upstream events including the processing and filter functions of the epithelium are in need of investigation. In the present work, we examined how BLG and processed forms thereof are transported across an *in vitro* reconstituted epithelial monolayer. Changes in immunoreactivity resulting from reduction/alkylation and tryptic digestion were determined by activation of BLG-responsive basophils. Together, our data show that the combination of the two approaches may represent an adequate screening assay to assess man-made hypoallergenic formulations and explore how commensals or food-grade bacteria can affect allergenicity.

Our results indicate that denaturation, enzyme hydrolysis and exposure to either wtLI or recLI strains lead to more efficient transepithelial transport of BLG across polarized Caco-2 cell monolayers (Fig. 4). Specifically, tryptic digestion was found to generate peptide products endowed with distinct transport properties, both in terms of rate and pathway. BLG and peptides T-6 and T-17 were transported via transcytosis and consequently could be further exposed to cathepsin proteases of the epithelial cell degradative pathway [33, 34]. In contrast, peptides T-

13 and T-24 followed the paracellular pathway. The functional outcome of these observations remains to be determined, with future paths of research focusing on features including presentation by intestinal epithelial cells [34, 35] and alteration of the nature of antigen transport during sensitization [9].

By modifying the structure of BLG using trypsin digestion or reduction/alkylation, we observed a pronounced decline in terms of IgG reactivity (Fig. 1). This was due to either exhaustive BLG cleavage or structural changes resulting from the breakdown of disulphide bridges. This is consistent with a previous study that demonstrated decreased interaction of reduced cyanogen bromide-derived and tryptic BLG fragments with rabbit seric IgG [36]. Incubation of BLG with recLI significantly reduced the immunoreactivity of the protein as well; as wtLI showed a milder effect, the processing mediated by lactic acid bacteria observed in our experiments could partly be attributed to the heterologous proteinase itself. Even though this observation does not allow to extrapolate to the global effect of the microbiota, it still underlines the possible contribution of luminal bacteria to the degradation of dietary proteins. This adds to the homeostatic properties of lactic acid bacteria known to stabilize intestinal permeability [5, 37, 38] and to the shaping of the mucosal immune system towards development of oral tolerance [39]. Together, our observations indicate that besides transformation of nutrients for efficient absorption, one of the main functions of intestinal processing is to reduce food immunoreactivity. In this respect, it is worth mentioning that BLG-specific IgG antibodies from allergic patients preferentially bind to the native form of the protein [40], a phenomenon that might partly be

attributed to incompletely processed proteins gaining access to the mucosal immunocompetent cells.

The contribution of intestinal epithelial cells to dietary protein processing is dual. Digestion by brush border peptidases represents the last step of luminal food antigen handling before uptake by enterocytes [41, 42], while additional intracellular endolysosomal processing occurs during transepithelial transport of BLG across rabbit ileum mounted in diffusion chambers [18, 43]. Our *in vitro* data provide evidence that in addition to acting as a selective filter for molecules, epithelial cells are capable of degrading BLG during transport. Based on the difference obtained between the ratios in Fig. 3c ($23 \pm 4\%$), the extent of epithelial cell processing was evaluated to be similar to the degree of BLGbiot alterations resulting from incubation with recombinant *L. lactis* ($23 \pm 10\%$; Fig. 2a), but remained markedly lower than the effects of denaturation or proteolysis ($85 \pm 3\%$ and $97 \pm 2\%$, respectively; Fig. 1d). Incidentally, the 1–3% of remaining immunologically intact form observed using the *in vitro* model correlates with the percentage of intact food antigen transported through the normal mature gut [44].

Several lines of evidence stress the importance of an efficient alteration/destruction of epitopic determinants of cow's milk proteins to avoid elicitation of an allergic immune response [45, 46]. In the case of BLG, our data indicate that trypsin hydrolysis or incubation with wtLI or recLI contribute to fulfilling this prerequisite, as demonstrated by the reduction of hexosaminidase released by IgE-sensitized basophils (Fig. 6). In contrast, reduction/alkylation of BLG intramolecular disulphide bonds only abrogated the reactivity of the protein towards IgG antibodies. This apparently paradoxical observation stresses an interesting point about structural bases of the immunoreactivity and allergenicity of BLG. This indicates that BLG-allergenic epitopes only partly overlap antigenic determinants, and suggests that IgE-binding sites are most likely restricted to parts of the molecule different from the IgG-binding sites, which are destroyed by disulphide bridge reduction [36, 47]. Similar to partial tryptic digestion that results in unmasking of hidden IgE epitopes [47, 48], denaturation per se does not appear to be sufficient to reduce BLG allergenicity significantly. Rather, the functional consequence of denaturation was reported to increase BLG digestibility and decrease the ability of the protein to elicit an allergic response upon oral challenge in a dog model of systemic sensitization [2].

We conclude that although the loss of immunogenicity of an antigen is indicative of conformational changes, it remains crucial to assess the absence of productive IgE cross-linking on target basophils or mast cells. In the physiological context, it is likely that IgG and IgE antibodies recognize less epitopic determinants on lumenally processed allergen than on the native form; however, reduced IgE reactivity can still bring about the cross-

linking of basophil/mast cell-bound IgE antibody molecules. Moreover, because little difference in BLG-IgG antibody specificity between cow's milk-allergic patients and control subjects was measured, it is essential to evaluate how IgE antibody specificity affects clinical reactivity [49]. In a similar context, clinical reactivity to peanut allergens is better analysed by determining epitope-specific binding than quantitative IgE values [50]. In addition, the antibody specificity depends on both structural characteristics of the antigen [51] and host factors such as immune response genes [52]. The biochemical and cellular models reported in this study represent a sound alternative to screen for allergen and derivatives before moving to more demanding *in vivo* trials including animal models and eventually human patients.

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