

Elicitation of the allergic reaction in β -lactoglobulin-sensitized Balb/c mice: biochemical and clinical manifestations differ according to the structure of the allergen used for challenge

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Summary

Background Mouse models of allergy are used to study the mechanisms of induction and perpetuation of bronchopulmonary hyper-reactivity (BHR) as related to eosinophils and specific IgE.

Objective Our aim was to adapt the current model for the study of bovine β -lactoglobulin (BLG), a major cow's milk allergen, and to further analyse the mechanisms of the acute and late allergic reaction.

Methods Female Balb/c mice were sensitized intraperitoneally with BLG and the influence of the adjuvant and of the BLG dose on the IgE response was analysed, IgE and IgG1 epitopes being characterized. Once optimized, this model was applied to the study of the active phase of allergy in the respiratory tract after a single airway challenge using native or denatured BLG, which contains only linear epitopes.

Results An immediate allergic reaction was characterized by the rapid release of histamine into the bronchoalveolar lavage fluids. Prostaglandin (PG)D₂ was only present when the standard histamine-releasing agent compound 48/80 or denatured BLG were used as triggers, whereas native BLG induced leukotriene release. Twenty-four hours after challenge, BHR, eosinophil influx, IL-4 and IL-5 production, plasma exudation and mucus production were very much increased, differently depending on the allergen structure, and indicated the occurrence of the late allergic reaction. Our results show that the murine model can be used to study the mechanisms of allergy to clinically relevant antigens, such as those contained in cow's milk. The acute allergic reaction, which depends on the structural feature of the allergen, is composed of two distinct pathways characterized by peptido-leukotrienes or PGD₂ production, which may result from distinct activation intensities of mast cells, leading to distinct late reactions.

Conclusion This study thus demonstrates a clear link between the structural feature of a protein, and the physiopathology of the experimental asthmatic reaction.

Keywords acute and late allergic reaction, bovine β -lactoglobulin, conformational/linear epitopes, IgE, leukotrienes/prostaglandins, mouse model, leukotrienes/prostaglandins

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Introduction

Allergy to cow's milk is essentially an IgE-mediated hypersensitivity reaction, although other immune mechanisms and clinical (delayed or late) reactions have also been identified [1]. Cow's milk allergy was diagnosed in 1.9–3% of the general population of infants in the first 2 years of life in various countries of Northern Europe [2]. The clinical picture of cow's milk allergy can vary from mild to severe, involving the skin, the gastrointestinal and respiratory tracts, as well as the cardiovascular

system (reviewed in [3]). Bovine β -lactoglobulin (BLG) is one of the major cow's milk allergen, and is therefore of paramount clinical importance [4]. The three-dimensional structure of BLG, maintained by two intramolecular disulphide bridges, resembles the human retinol-binding protein and the other proteins of the lipocalin family [5] which share the property of binding small hydrophobic molecules within the central cavity of the β -barrel of the calyx [6]. IgE from patients allergic to BLG were shown to be specific both for native and denatured BLG (without the intramolecular S–S bridges, and accordingly without conformational epitopes), in variable proportions [7]. This suggests the implication of both linear and conformational epitopes in the allergic reaction to BLG in humans. Interestingly, conformational versus linear epitopes have recently been shown to be differentially involved in the achievement of tolerance in cow's milk allergy. Patients with persistent milk allergy

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were shown to possess higher levels of IgE specific to linear epitopes from α s1 and β -casein than children who have achieved tolerance [8]. This suggests a differential role for linear/conformational epitopes in humans.

Genetic analysis have shown that allergen-induced bronchopulmonary hyper-reactivity (BHR) and IgE levels are closely related [9]. Antigen–antibody-mediated cross-linking of IgE receptors on the surface of mast cells and their resulting activation are considered important for the initiation and development of asthma [10], but may play a less prominent role in its chronic phase. Following IgE-induced mast cell degranulation via interaction with Fc ϵ RI, pre- or newly synthesized mediators are released, which lead to bronchial smooth muscle constriction, oedema and mucus hypersecretion [11], followed by the synthesis of cytokines as IL-4, IL-5 and IL-13 [12]. These cytokines induce mast cell, eosinophil and T helper type-2 (Th2) cell development as well as mucosal metaplasia, thus amplifying and promoting the local allergic response.

Over the past several decades, it has become clear that innovative animal studies can contribute greatly to define the mechanisms of asthma. However, experiments using murine models provided conflicting results concerning the implication of IgE in airway reaction. Passive sensitization with ovalbumin IgE and IgG1 and subsequent challenges led to increased airway responsiveness and to the influx and activation of eosinophils to lung tissue [13]. Similar results were obtained using actively immunized BP2 mouse [14]. The importance of free IgE and allergen–IgE complexes in IL-4 production and eosinophils influx have also been demonstrated [15]. By contrast, other studies suggested that IgE are not implicated, but the role of IgG1 was not evaluated in those models [16, 17].

Because of the medical importance of allergy to milk proteins and the need of an animal model to study its mechanisms, we decided to adapt the model of asthma, as developed in ovalbumin (OVA)-immunized mice, particularly BP2 [14], to Balb/c mice immunized with BLG. BHR in Balb/c mice has been suggested to be regulated by the IgE-mediated activation of mast cells, whereas it depends exclusively on IL-5 and eosinophils in C57BL/6 mice, which are deficient in mast cell protease [18, 19]. BP2 mice, which display more intense airway responses compared to Balb/c, have been used previously, IgE and BHR being closely linked [14]. However, Balb/c mice, which develop a Th2-biased immune response [20], have been used successfully after multiple intranasal (i.n.) challenges. Optimization of specific IgE response in these mice thus appears as an essential first step for our purpose. We previously developed enzyme immunoassays allowing the quantification of BLG-specific IgE, IgG1 and IgG2a in BLG-sensitized mice [21]. In the present paper, we used these tools to study the induction and optimization of anti-BLG IgE response in Balb/c mice, and to characterize the induced response. The functionality of such a model to study the immediate and delayed phases of allergy in the respiratory tract was then evaluated. For this purpose, several important parameters observed in human asthma were measured: (i) allergen-specific IgE in serum of immunized mice; (ii) histamine, leukotriene (LT) and prostaglandin (PG) releases in the first minutes after allergen challenge; and (iii) BHR, Th2 cytokines (IL-4 and IL-5) in BAL fluids, eosinophil infiltration 24 and/or 72 h after the allergen challenge. We observed that those reactions were different, depending on whether native or denatured BLG were used for challenge, thus demonstrating the

important and differential role of conformational versus linear epitopes. Moreover, our results further support the implication of specific IgE and IgG1 in the initiation of asthma-like reaction in mice.

Material and methods

Apparatus

Enzyme immunometric assays were performed in 96-well microtitre plates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) using specialized Titertek microtitration equipment from Labsystems (Helsinki, Finland).

Reagents

Unless otherwise stated, all reagents were of analytical grade from Sigma (St Louis, MO, USA). BLG variant A and reduced and carboxymethylated BLG (rcmBLG) were prepared as previously described [4].

Quantification of BLG-specific IgE, IgG1 and IgG2a

These assays are described in detail elsewhere [21]. BLG was indirectly immobilized via streptavidin–biotin interaction; this coating allows the detection of antibodies binding both denatured and native forms of BLG. Quantification of specific IgE is preceded by removal of serum IgG using Protein G immobilized on porous glass (PROSEP, Bioprocessing, Consett, UK), to avoid IgG interference in IgE detection, especially during secondary and late response. Specific IgE concentrations are corrected using a loss factor 'R' (R = total IgE before protein G treatment/total IgE after protein G treatment), allowing calculation of actual concentrations. This procedure was validated in our laboratory, and allows the authentic quantification of all specific IgE whatever the IgG concentrations.

Analysis of the specificity of the different anti-BLG isotypes produced was performed on BLG-biot- and rcmBLG-coated plates, as described [21]. Epitope specificity was analysed using AChE-labelled synthetic peptides and anti-isotype coated plates, as previously described [22].

Mice immunizations

Female Balb/c mice were from CERJ (Centre d'Élevage Rene Janvier, Le Genest Saint-Isle, France), and were housed under normal husbandry conditions. Mice were acclimated for 2 weeks before immunizations, and were used when 6 weeks old. The absence of BLG in mice feed was verified using a sensitive sandwich immunoassay [23]. In a first set of experiments, mice were immunized intraperitoneally (i.p.) on day 0 with 50 μ g of BLG adsorbed onto alum (Alhydrogel 3%, Suprefos Biosector a/s, Denmark) used at 1 mg/mouse, or emulsified with Complete Freund's Adjuvant (CFA, Difco Laboratories, Detroit, MI, USA) in a 1 : 1 ratio. Injected volume was 0.2 mL/mouse. A booster injection was performed on day 21 in alum or with Incomplete Freund's Adjuvant (IFA), respectively. In a second set of experiments, various doses (0, 0.5, 5, 50 or 250 μ g) of BLG were used to optimize anti-BLG IgE response, and booster injections at 14 days post-sensitization were also evaluated.

In some experiments, positive controls were made using mice immunized on days 0 and 14 with 10 µg OVA adsorbed onto alum (1 mg/mouse), and further challenged i.n. with OVA.

Immediate release of the mediators of the active phase of allergy

Challenge and BAL collection On day 24, mice were anaesthetized by intravenous (i.v.) injection of ketamine (35 mg/kg, Imalgène, Merial, Lyon, France). The trachea was dissected, and mice were intratracheally challenged with 10 µg of native BLG (BLGn) or rcnBLG, considered as denatured BLG (BLGd), in 50 µL saline. Negative control groups corresponded to BLG-immunized mice challenged with 10 µg of OVA. Positive control groups comprised immunized mice challenged with 50 µg of the standard histamine-releasing agent, compound 48/80. One, 3 or 10 min after provocation, bronchoalveolar lavage (BAL) fluids were collected in 3 × 0.5 mL NaCl 0.9%, and immediately filtered (Millex® – SV, 5.0 µm filter unit, Millipore, Molsheim, France) to remove cell debris. The first BAL fluids collected, corresponding to approximately 0.3 mL of fluids, were filtered above 30 µL PCA 1 M, mixed and immediately frozen using liquid nitrogen. Aliquots were stored at –80 °C until histamine assay. The two BAL fluids collected next were pooled after filtration, and immediately frozen using liquid nitrogen. Those aliquots were stored at –80 °C until LT, PG and albumin assays.

Histamine assay Histamine was assayed by competitive enzyme immunoassay as described [24]. One hundred µL of a biotinylated antihistamine monoclonal antibody (mAb31, 0.1 µg/mL) was immobilized on neutravidin-coated plates (5 µg/mL, 150 µL/well in 50 mM phosphate buffer pH 7.4). Before assay, standard and samples in PCA 0.1 M were derived using Bolton–Hunter reagent: 100 µL of standard or samples were mixed with 50 µL of derivation buffer (NaOH/borate 0.5 M, pH 11), and 20 µL of Bolton–Hunter reagent (50 mg/mL in DMF) for 1 h reaction at 20 °C. Then, 75 µL of derived standard or samples and 150 µL of enzyme tracer (AChE-labelled histamine-SATH), were dispatched to the mAb31-coated wells. After an 18-h incubation at 20 °C, plates were washed and immobilized AChE was revealed using Ellman's reagent. mAb31 and tracers used were kindly provided by SPI-BIO (Massy, France).

Eicosanoid assays Competitive enzyme immunoassays were performed, using AChE-labelled eicosanoids, as previously described for LTB₄, LTC₄, LTE₄ [25], PGE₂ and PGD₂ [26]. The reagents (antisera and tracers) used in these assays were kindly provided by SPI-BIO.

Evaluation of bronchopulmonary hyper-reactivity and mucus production

Hyper-reactivity 24 h after i.n. challenge On day 24, BLG-immunized mice were challenged i.n. with 10 µg of BLGn, BLGd, or OVA (negative control), or with 50 µg of compound 48/80. Fifty µL of saline containing the chemicals were instilled i.n. under light anaesthesia (Imalgène, 25 mg/kg), with the aid of a micropipette. On day 25, unrestrained, conscious mice were placed in a plethysmographic chamber (Buxco Electronics,

Sharon, CT, USA) and respiratory parameters were measured before (–3 to 0 min) and after (1–10 min) exposure to 100 mM aerosolized methacholine for 20 s. Enhanced pause (Penh) was taken as an index of airway obstruction [27], and calculated according to the manufacturer's recommendations. Mice were then deeply anaesthetized by the i.p. injection of urethane (15 mg/10 g body weight), and the trachea was cannulated for bronchoalveolar cells collection in sterile saline. Cells were counted with a Coulter Counter ZM (Coultronics, Margency, France). Differential cell counts were performed after cytocentrifugation and staining with Diff-Quick stain (Baxter Dade AG, Duedingen, Switzerland). Aliquots of the remaining lavage fluids were centrifuged and stored at –80 °C until cytokine assays. Those assays correspond to specific sandwich ELISA (see above).

In some mice, differential cell counts were performed 72 h after i.n. challenge.

Cytokine assays IL-4 was measured by enzyme immuno-metric assay. Briefly, microtitre plates were coated with 2 µg/mL of rat anti-mouse IL-4 (BVD4-1D11; Pharmingen, San Diego, CA, USA), to which were added dilutions of recombinant IL-4 standard (7–1000 pg/mL) or samples, followed by an addition of biotinylated rat anti-IL-4 antibody (BVD6-24G2; Pharmingen) at 0.5 mg/mL. The reaction was revealed using a substrate containing *o*-phenylenediamine dihydrochloride in phosphate buffer, and colouration was measured at 490 nm. IL-5 was measured using an immunometric assay as previously described, using rat anti-mouse IL-5 monoclonal antibodies TRFK-4 and TRFK-5 [14].

Evaluation of lung eosinophil peroxidase activity To quantify the lung sequestration of eosinophils, eosinophil peroxidase (EPO) activity was evaluated in 96-well plates using a cytochemical enzyme assay, based on the oxidation of *o*-phenylenediamine by EPO in the presence of hydrogen peroxide. This assay is carried out in the presence or absence of the EPO inhibitor 3-amino-1, 2, 4-triazole [28].

Mucus production In four mice of each group, mucus production in lungs was studied 72 h after i.n. challenge. Mice were anaesthetized and the trachea was cannulated. Lungs were washed and perfused via the right ventricle with 10 mL saline to remove residual blood. Then, 1 mL of 10% phosphate-buffered formalin was injected via the cannula, trachea was ligatured and lungs were removed. They were immersed in the same buffer until they were sliced and 5-µm sections were stained with haematoxylin/eosin for light microscopy examination or with periodic acid–Schiff (PAS)/haematoxylin for the evaluation of mucus-producing cells. Mucus production was determined by counting the number of PAS-positive bronchi or the number of bronchi containing plugs of mucus.

Statistical analyses

Statistical analyses were conducted with GraphPad software. Data were analysed using analysis of variance (ANOVA) and Dunett's test when comparing treated mice with control group, or using ANOVA and Tukey's test when comparing all the groups each others.

Results

Elicitation of a high anti-BLG IgE response in Balb/c mice

In a first set of experiments, we evaluated the anti-BLG IgE, IgG1 and IgG2a responses in mice sensitized twice i.p. with 50 µg BLG either adsorbed onto alum, or emulsified with CFA/IFA. Although an intense primary response was only observed in alum-immunized mice, the two immunization protocols were shown to induce nearly equivalent concentrations of specific IgE during second and late responses. Alum mainly induced antibodies of the IgE and IgG isotypes directed against the native form of the molecule (i.e. conformational epitopes), with 15–30% of antibodies specific for a denatured form of BLG. Those antibodies have been shown to recognize only the N-terminal sequence of BLG, mainly synthetic peptides (11–26), (21–40) and (41–60) [22]. Conversely, CFA/IFA-induced antibodies mainly directed against the denatured form of the antigen, whatever the isotype. Linear epitopes recognized by IgE and IgG1 correspond to synthetic peptides (11–26), (21–40), (41–60), (120–135) and slightly to (102–124).

In other experiments, IFA was used in place of CFA, which contains DNA from *Mycobacterium butyricum* that may polarize the response towards a Th1-type response [29]. A higher IgE response was noted with IFA/IFA as compared to CFA/IFA (data not shown). A dose–response experiment using IFA or alum was then performed to further optimize the IgE response. For both adjuvants, 5 µg of BLG proved to be optimal for inducing specific IgE.

These preliminary experiments allowed us to select a protocol using 5 µg of native BLG adsorbed onto alum (1 mg/mouse) which was administered i.p. in order to induce intense anti-BLG IgE responses. A last experiment included the booster injection on day 14, as the intensity of the IgE response was nearly equivalent to that obtained when it was performed on day 21. This model of elicitation of specific IgE was applied to the study of the active phase of allergy, by evaluating the immediate release of various mediators following the intratracheal (i.t.) challenge with BLG, and bronchial responsiveness 24 and 72 h after an i.n. challenge. The following results are

representative of at least two independent experiments with at least six animals each.

Evaluation of the active phase of allergy in BLG-sensitized mice

A total of 128 mice were immunized on days 0 and 14, and sera were collected on day 21. Mean values for anti-BLG IgE, IgG1 and IgG2a were 355.8 ± 118.3 ng/mL, 244.1 ± 121.9 µg/mL and 496.4 ± 360 ng/mL, respectively. Mice were then randomly separated into four homogenous groups of 32 mice each, which were challenged with ovalbumin, BLGn, BLGd or compound 48/80 on day 24, corresponding to a maximal IgE response.

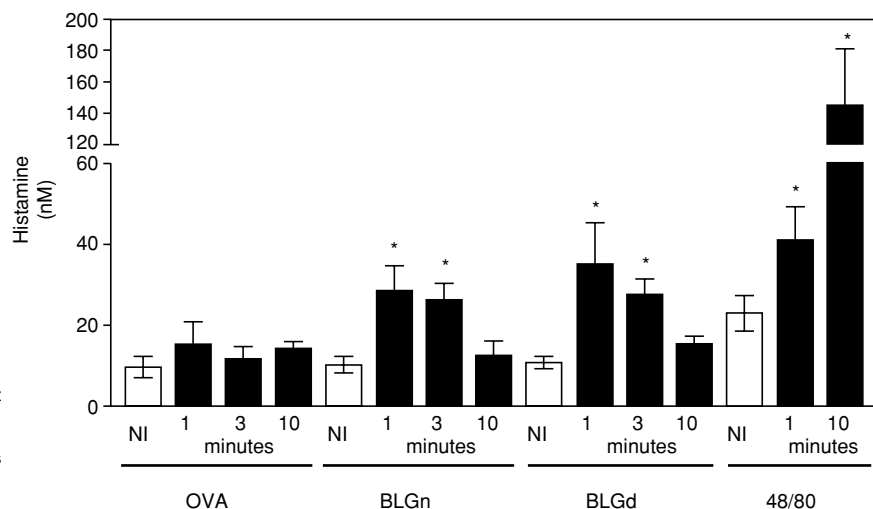
Mediator release in BAL fluids 1, 3 and 10 min after i.t. challenge

Histamine Non-immunized mice were challenged i.t. with OVA, BLGn, BLGd and compound 48/80 as a positive control for histamine release, and histamine release was measured after 1 min (Fig. 1, open bars). Release in those mice corresponds to background values due to manipulation, except for compound 48/80 where additional histamine release was observed. In BLG-sensitized mice, histamine release was significant 1 and 3 min after challenge with BLGn and BLGd. Positive control (challenge with compound 48/80) allowed for an equivalent release at 1 min. Already 10 min after challenge, no significant histamine release was observed in BLGn- or BLGd-challenged mice, whereas it further increased in compound 48/80-challenged mice.

LT and PG productions LTC₄ (Fig. 2a) was only produced in BLGn-challenged mice, 3 min after the i.t. provocation. LTC₄ (Fig. 2b) and LTE₄ (Fig. 2c) productions increased with time for BLGn-challenged mice, to reach a value close to 400 pg/mL at 10 min. Very low amounts were detected at this time point in BLGd- and 48/80-challenged mice.

PGD₂ was present in sensitized mice 10 min after the i.t. challenge with BLGd or compound 48/80 (Fig. 3). Contrary to leukotrienes, BLGn did not induce a significant PGD₂

Fig. 1. Histamine release in BAL fluids 1, 3 and 10 min after intratracheal challenge: on day 24, BLG-in-alum-immunized mice were anaesthetized and then intratracheally challenged with OVA (negative control), BLGn, BLGd, or compound 48/80. BAL fluids were collected 1, 3 or 10 min later, filtered above PCA and frozen until histamine assay. Non-immunized mice were also challenged with those compounds and BAL fluids were collected 1 min later (open bars). Mean of eight mice per group $\pm$ SEM are represented. *Indicates a significant difference ($P < 0.05$) using analysis of variance (ANOVA) and Dunnett's test (control group: non-immunized/challenged mice – 1 min).



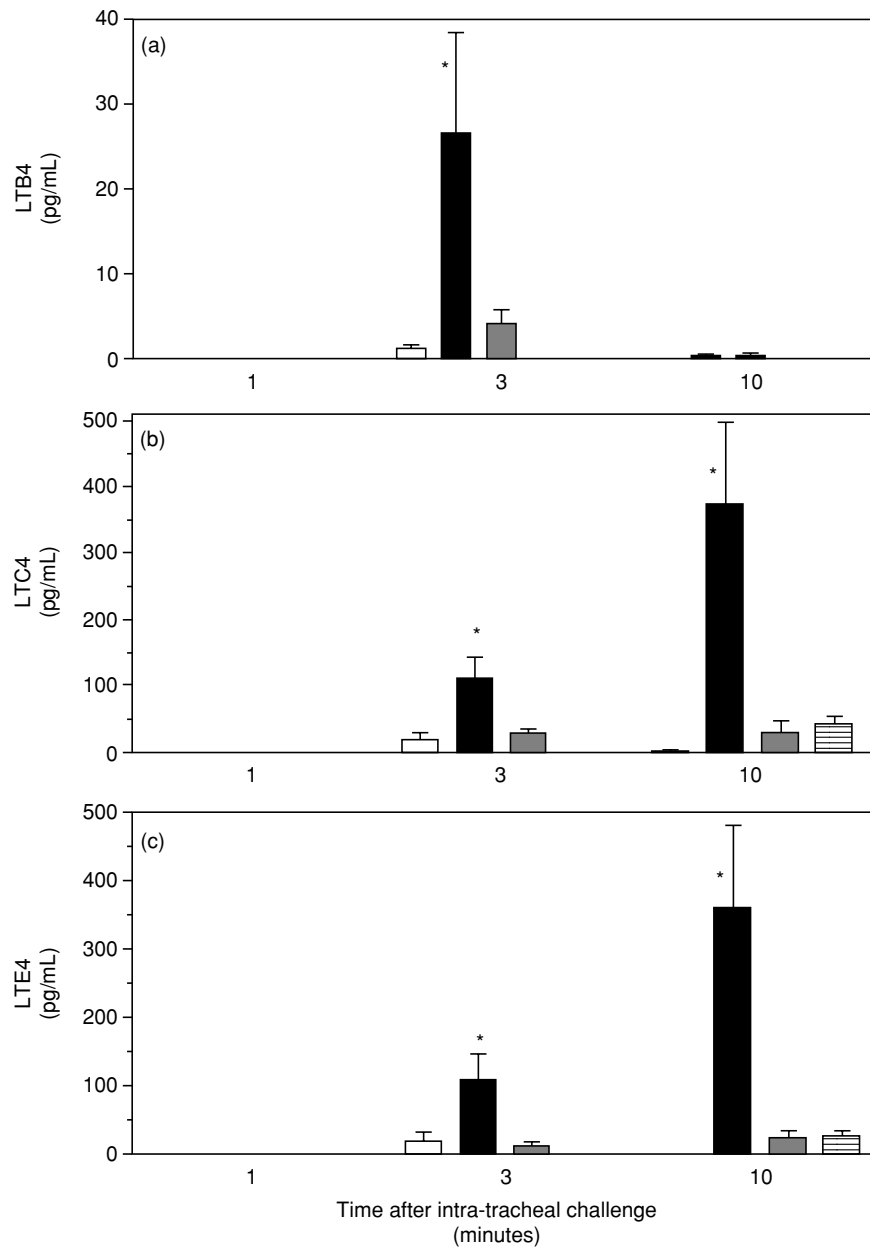


Fig. 2. Leukotriene release in BAL fluids 1, 3 and 10 min after intratracheal challenge: on day 24, BLGn-in-alum-immunized mice were anaesthetized and then intratracheally challenged with OVA (negative control, white bars), BLGn (black bars), BLGd (grey bars), or compound 48/80 (hatched bars). BAL fluids were collected 1, 3 or 10 min later, and then immediately filtered and frozen until leukotriene assays. LTB4 (a), LTC4 (b) and LTE4 (c) assays were performed as described in the Methods section. Mean of eight mice per group $\pm$ SEM are represented. *Indicates a significant difference ($P < 0.05$) between BLGn-challenged mice and other groups, using ANOVA and Tukey's test.

production when compared to OVA challenge. PGE2 was not detected in the different groups.

BHR and cellular events 24 and 72 h after the i.n. challenge of sensitized mice

BHR BHR was monitored as increased enhanced pause (Penh) in conscious unrestrained mice placed in a whole-body plethysmograph, 24 h after i.n. challenge. Baseline was first monitored during 3 min, then methacholine was delivered by aerosol for 20 s.

Penh before methacholine inhalation was higher in compound 48/80-challenged mice, reflecting a constrictor effect of this compound (Fig. 4, $P < 0.001$ for 48/80-challenged mice vs. others groups). This effect was enhanced by methacholine. In BLGn-challenged mice, a slight BHR appeared rapidly after

methacholine (from 1 to 4 min), as compared to control mice challenged with OVA, but differences are not significant ($P > 0.05$) and did not persist. BHR was more intense in BLGd-challenged mice ($P < 0.05$ for BLGd challenged vs. OVA-challenged mice at time 2, 3, 4 and 5 min), and was not statistically different from that found in OVA-immunized and challenged mice, except at times 1 and 2 min.

Cytokine production 24 h after challenge IL-4 was detected only in BLGn-challenged mice ($52.7 \text{ pg/mL} \pm 40$, mean of eight mice per group $\pm$ SEM, $P < 0.05$ for BLGn vs. BLGd- or OVA-challenged mice using ANOVA and Tukey's test). IL-5 was detected in both BLGn- and BLGd-challenged mice ($50.5 \text{ pg/mL} \pm 19.1$ and $50 \text{ pg/mL} \pm 27.3$, respectively, mean of eight mice per group $\pm$ SEM; $P < 0.05$ for BLGn- and BLGd- vs. OVA-challenged, ANOVA and Tukey's test).

Cell influx Eosinophil influx in BAL fluids was detected only in BLG-immunized mice, 24 and 72 h after i.n. challenge with BLGn (Fig. 5). This influx was less intense than after challenge in OVA-sensitized mice. Neutrophils and lymphocytes were recruited in BLGn-, BLGd- and 48/80-challenged mice.

EPO measurements EPO in lung tissue was measured as a marker of eosinophil sequestration, 24 h after the i.n. challenge. EPO values were 0.0185 ± 0.025 in OVA-challenged mice, 0.068 ± 0.018 in BLGn-challenged mice and 0.06 ± 0.023 in BLGd-challenged mice ($P < 0.05$ for BLGn vs. OVA and BLGd vs. OVA, ANOVA and Newman–Keuls multiple comparison test). Thus, eosinophil influx into lung occurred in both BLGn- and BLGd-challenged mice. However, eosinophils in BAL were observed only in BLGn-challenged mice.

Mucus production and inflammation 72 h after challenge In BLGn-challenged mice, mucus production (Fig. 6b) and eosinophil infiltration (Fig. 6d) were significant when compared

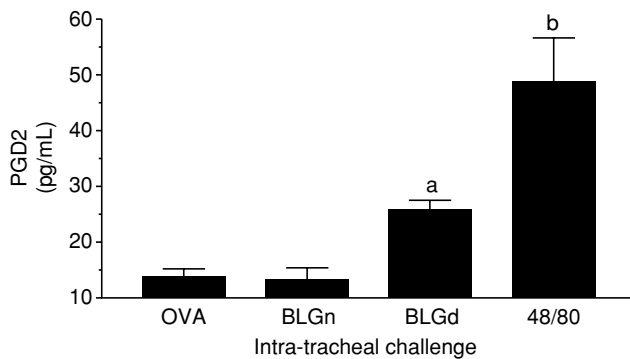


Fig. 3. Prostaglandin concentration in BAL fluids 10 min after the intratracheal challenge: on day 24, BLG-in-alum-immunized mice were anaesthetized and then intratracheally challenged with OVA (negative control), BLGn, BLGd, or compound 48/80. BAL fluids were collected 10 min later and immediately filtered and frozen until assays. PGD2 assays were performed as described in the Methods section. Mean of eight mice per group $\pm$ SEM are represented. 'a' indicates a significant difference of BLGd groups versus other groups ($P < 0.05$) and 'b' indicates a significant difference of 48/80 groups versus other groups ($P < 0.01$), using ANOVA and Tukey's test.

to OVA-challenged negative control mice (Figs 6a and c, respectively) or 48/80-challenged mice (data not shown). Mucus production and eosinophilic infiltration in lungs were also observed in mice challenged with BLGd (data not shown).

Discussion

In this study, we describe the induction and optimization of anti-BLG responses in Balb/c mice, and the use of such a model to study the immediate and delayed phases of allergy in the respiratory tract after challenging with the native or the denatured proteins via the airways. We evaluated several parameters observed in human asthma: (i) allergen-specific IgE; (ii) leukotriene, prostaglandin and histamine releases during the first minutes after challenge; and (iii) BHR, Th2 cytokines (IL-4 and IL-5 in BAL fluids), and eosinophil infiltration 24 and/or 72 h after challenge.

In a first set of experiments, we studied the mediator release 1, 3 and 10 min after the i.t. provocation with native or denatured BLG, in mice sensitized with native BLG. Native BLG, containing conformational epitopes, triggered the release of histamine, LTB₄, LTC₄ and LTE₄, whereas denatured BLG, containing only linear epitopes, led to the release of histamine and PGD₂, with virtually no LTs. Further experiments demonstrated a clear dichotomy between the immediate release of mediators and BHR. In BLGn-challenged mice, a slight BHR was observed in the first minutes after methacholine inhalation, and a significant and marked eosinophil influx into BAL and lung tissue, which correlated with IL-5 release and LT production. However, an intense BHR was observed in BLGd-challenged mice, which did not correlate with eosinophil influx into BAL fluids. This absence did not result from failure to produce IL-5, as those mice showed IL-5 titres in BAL comparable to those of BLGn-challenged mice. Eosinophil absence may be one of the final consequences of the lack of early production of LTs, which have been shown to be a chemoattractant for eosinophils *in vitro* [30] and *in vivo* (Vargaftig and Singer, in press). The other difference between BLGn- and BLGd-challenged mice was the early PGD₂ synthesis in the

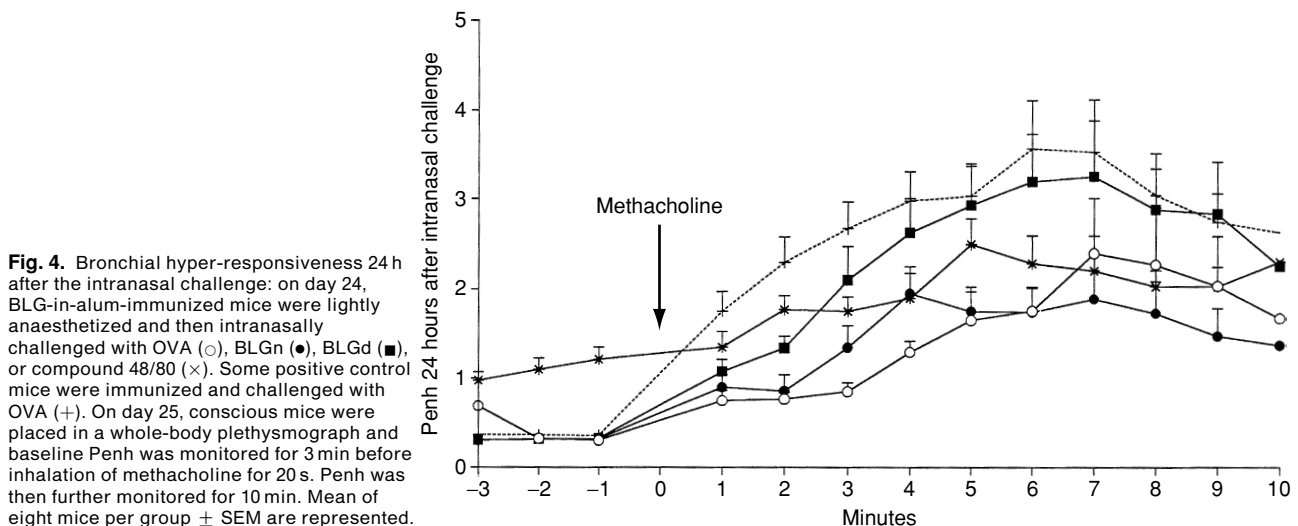


Fig. 4. Bronchial hyper-responsiveness 24 h after the intranasal challenge: on day 24, BLG-in-alum-immunized mice were lightly anaesthetized and then intranasally challenged with OVA (○), BLGn (●), BLGd (■), or compound 48/80 (×). Some positive control mice were immunized and challenged with OVA (+). On day 25, conscious mice were placed in a whole-body plethysmograph and baseline Penh was monitored for 3 min before inhalation of methacholine for 20 s. Penh was then further monitored for 10 min. Mean of eight mice per group $\pm$ SEM are represented.

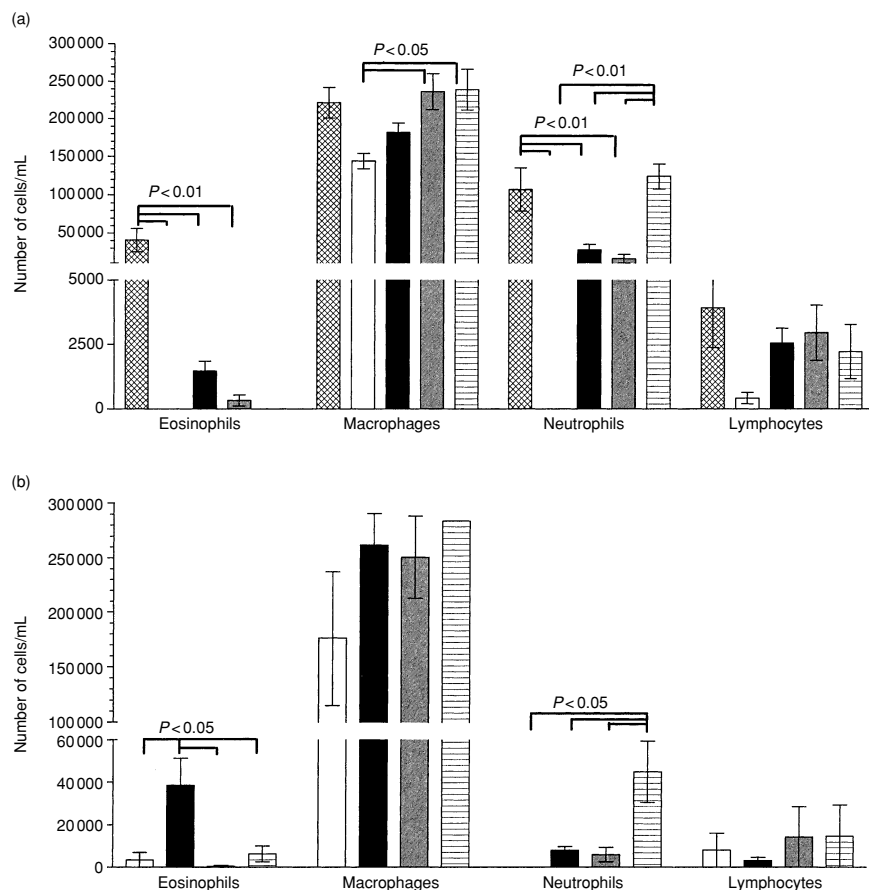


Fig. 5. Differential cell counts in BAL fluids 24 h after intranasal challenge: on day 24, BLG-in-alum-immunized mice were slightly anaesthetized and then intranasally challenged with OVA (□), BLGn (■), BLGd (▒), or compound 48/80 (▨). Some positive control mice were immunized and challenged with OVA (▨). Twenty-four hours (a) or 72 h (b) later, BAL fluids were collected, cytocentrifuged and stained with Diff-Quick stain. Mean of four (72 h) or eight (24 h) mice per group $\pm$ SEM are represented. *P*-values using ANOVA and Tukey's test are shown.

latter. This acute PGD2 synthesis was also found in compound 48/80-challenged mice, who showed increased airways resistance irrespective of methacholine inhalation. In fact, PGD2 has been previously shown to be important for allergic BHR, as a PGD2-receptor knockout mouse failed to develop it [31]. It is notable that BHR failed to correlate with EPO activity, as both BLGn- and BLGd-challenged mice showed increased EPO titres of the lung tissue.

It is likely that in the case of a single allergen challenge, as in our experiments, the early response determines the late one, and follows the binding of BLG to mast cell-bound IgE and/or IgG1, as mast cells were observed in the lungs of Balb/c mice [32]. Our results suggest two alternative mechanisms for the acute allergic reaction, depending on the intensity of mast cell activation, as no eosinophils or lymphocytes were present at the time of challenge, as seen in control mice (BLG-sensitized and OVA-challenged). A marked activation of mast cells may be induced by BLGn challenge. Because alum-immunized mice showed essentially IgE and IgG1 specific for the native form of BLG, the BLGn-IgE and/or BLGn-IgG1 recognition may be determinant and insures an intense mast cell activation, with histamine release and LT production. LT release would further promote eosinophil influx (Vargaftig and Singer, submitted). IL-4 found in BAL 24 h after i.n. challenge with BLGn may result in this intense activation of mast cells, and would participate in further activation of Th2 cells. This is supported by results demonstrating IL-4 production and eosinophils influx after intense mast cell activation in Balb/c mice [15]. In case of a

weaker activation, such as in BLG in alum-immunized and BLGd-challenged mice, where BLGd [and then linear epitopes (11–26), (21–40) and (41–60)] specific IgE and IgG1 titres are lower, the resultant activation of mast cells would only lead to histamine release and PGD2 production. The latter would further induce BHR via Th2 cell activation, and to late eosinophil influx into lung [31]. This is supported by the fact that IgE and IgG1 specific for denatured BLG are induced when mice are immunized by BLG emulsified in IFA. When those mice are challenged i.t. with BLGn, no LT production occurred, whereas challenge with BLGd led to elevated production of peptidoleukotrienes and to eosinophil sequestration (data not shown).

Our study highlights the differential role of conformational versus linear epitopes in the elicitation of the active phase of allergy with BLG, depending on the specificity of the antibodies induced. Interestingly, conformational versus linear epitopes have recently been shown to be differentially involved in the achievement of tolerance during cow's milk allergy. Patients with persistent allergy showed higher titres of IgE specific to linear epitopes from α s1 and β -casein as compared to children who had achieved tolerance [8]. Those results may suggest that the discrepancies observed in sensitized mice, depending on the structural form of the allergen, may occur in allergic humans. Moreover, this further demonstrates the usefulness of the present model, in studying the early events of the reaction after a single airway challenge and in the establishment of a clear link between the structural feature of a protein, and the pathophysiology of the asthmatic reaction.

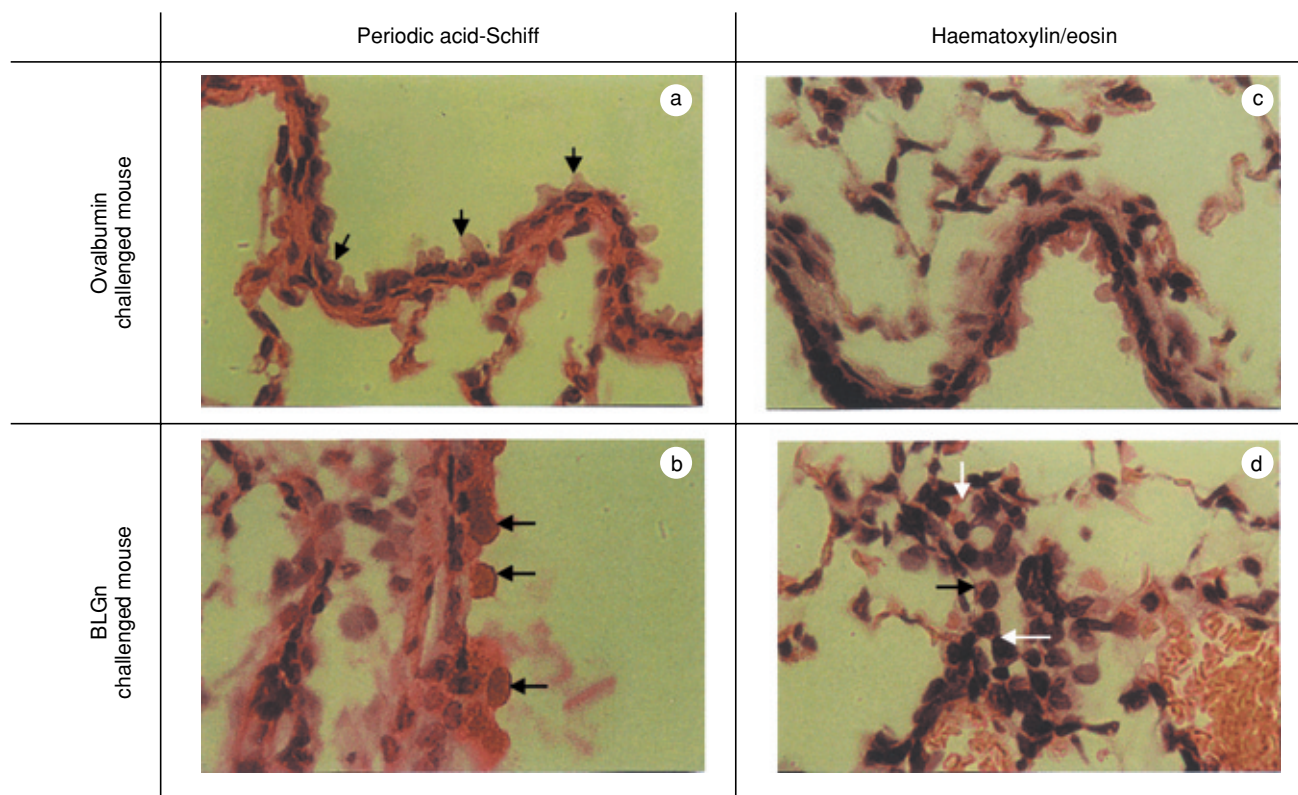


Fig. 6. Mucus-producing cells and eosinophil influx in BLGn-challenged mice: on day 24, BLGn-in-alum-immunized mice were lightly anaesthetized and then intranasally challenged. Seventy-two hours later, lungs were removed, fixed in formalin and sliced. Then, 5- μ m sections were stained with periodic acid-Schiff [a: OVA-challenged mice (arrows show PAS-negative epithelial cells), b: BLGn-challenged mice (arrows show PAS-positive epithelial cells)] or with haematoxylin/eosin [c: OVA-challenged mice (no peribronchial inflammatory infiltration), and d: BLGn-challenged mice (arrows show intense peribronchial/perivascular eosinophil/mononuclear cell infiltration)]. Original magnification $\times 400$. Lung sections from representative mice are shown.

Our work underlines the role of IgE and IgG1 after a single i.n. challenge for the development of the immediate active phase of allergy, and for the late response (eosinophils, BHR) in Balb/c mice. This supports previous reports in mice using neutralizing anti-IgE antibodies [32], Stat6-deficient mice [33], or passive sensitization with IgE or IgG1 anti-OVA antibodies [13]. Corry et al. [18] have also shown that, although a contributory role for eosinophils for chronic airway changes in late-phase asthmatic response is not excluded, IL-5 and eosinophils are not involved in the acute airway hyper-reactivity in Balb/c mice. Proust et al. (manuscript submitted) have obtained similar data for Balb/c and BP2 mice.

Mucus production was observed both in BLGd- and BLGn-challenged mice, supporting previous results showing that both LTs [34] and PGD2 [31] are involved in the induction of mucus production. Moreover, the results obtained in BLGn-immunized and -challenged mice confirms that BHR is independent of mucous-cell metaplasia, as previously shown [32].

The present model of IgE-induction and active allergy may be extended. First, modification of the BLG structure and its direct effects on the modulation of sensitization and elicitation phases of allergy may be evaluated, further demonstrating the interest of the BLG model in asthma studies. The same may be achieved using modified recombinant allergen or allergen fragments. Those studies may allow characterization of the linear and conformational epitopes involved in the elicitation of the

allergic reaction. Second, this model can also be used to determine if inhibition of IgE and IgG1 responses in mice is sufficient to induce an inhibition of T cells-related events such as BHR and mucus production. Consequences of IgE and IgG1 inhibition and of Th1 cell induction on early and late events will also be appreciated in DNA-based vaccination protocols [22].

Asthma chronification after repeated allergen challenges in Balb/c mice may involve early IgE-relayed by CD4⁺ T cells mechanisms. In fact, passive sensitization with IgE leads to weaker eosinophil recruitment than in actively sensitized Balb/c mice, probably because of a lessened involvement of cytokines, particularly from activated Th2 cells [13]. Moreover, Balb/c mice were shown to have two distinct pathways that are regulated by CD4⁺ T cells, which operate independently and in parallel. The first consists of IL-5-regulated eosinophilia and is critical for aeroallergen lung damages. The second regulates BHR and is not associated to lung damages [35]. Accordingly, BHR can develop independently of IL-5 and eosinophilia. Both pathways might result from the different mechanisms of acute activation of mast cells, IL-4 and LT productions in the first case, with intense eosinophil influx and no BHR, or PGD2 synthesis in the latter, with BHR. When airway challenges are multiplied or intensified, the IgE pathway is bypassed and CD4⁺ T cells are directly activated at the allergenic site. Consistent with this hypothesis, excessive challenge with allergen overcomes the effect of PGD2 receptor deficiency in the Matsuoka model [31].

In summary, using BLG as a model allergen, we developed and optimized a specific IgE-responder mouse model. This model was applied to the study of the active phase of allergy in the respiratory tract after a single airway challenge. Two alternative mechanisms of the acute allergic reaction were triggered in the initial minutes following challenge, depending on the structural form of the allergen and on the IgE and IgG1 binding capacity, demonstrating the relation between the structural feature of a protein and the physiopathology of the asthmatic reaction. These pathways are characterized by the production of peptido-leukotrienes or PGD₂, respectively, and may result from distinct activation intensities of mast cells, leading to distinct late reactions 24/72 h after challenge.

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