

Evaluation of a high IgE-responder mouse model of allergy to bovine β -lactoglobulin (BLG): development of sandwich immunoassays for total and allergen-specific IgE, IgG1 and IgG2a in BLG-sensitized mice

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Abstract

An animal model of food allergy represents an important tool for studying the mechanisms of induction and repression of an allergic reaction, as well as for the development of an immunotherapy to prevent or minimize such an adverse reaction. IgE and IgG1 (Th2 response) vs. IgG2a (Th1 response) are good markers for the induction of an allergic response in mice. Nevertheless, while the total serum concentrations of these isotypes are easy to measure using classical sandwich immunoassays, this is not the case for allergen-specific isotypes. To develop an animal model of allergy to bovine β -lactoglobulin (BLG), we set up quantitative assays for total and for allergen-specific IgE, IgG1 and IgG2a. Microtiter plates coated either with anti-isotype antibodies (Abs) or with allergen were used for Ab capture, while anti-isotype Fab' fragments coupled to acetylcholinesterase were used for visualization. These assays of anti-BLG specific Abs are original in two ways. First, assay calibration is performed using anti-BLG specific mAbs, thus allowing good quantification of the different isotypes and subclasses of serum antibodies. Second, the detection of all anti-BLG specific Abs, i.e., those recognizing both the native and denatured forms of the protein, is achieved through indirect coating of BLG using biotin–streptavidin binding. The present assays are quantitative, specific to the isotype (cross-reactivity < 0.5%), very sensitive (detection limit in the 10 pg/ml range), and reproducible (coefficient of variation less than 10%). Applied to the humoral response in mice sensitized with BLG adsorbed on alum, these assays proved to be a very useful tool for monitoring high IgE-responder mice following BLG immunization, and for an immunotherapy directed at polarizing the immune response. © 2000 Elsevier Science B.V. All rights reserved.

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Abbreviations: BLG: Bovine β -lactoglobulin; biotBLG: Biotinylated BLG; AChE: Acetylcholinesterase; total IgX: Total immunoglobulins of the X isotype; specific IgX: BLG-specific immunoglobulin of the X isotype; Alum: Al(OH)₃ gel

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1. Introduction

Atopy affects 20 to 30% of the population in industrial countries, while 3 to 5% present with a food allergy (e.g., immunologically-mediated reaction against dietary antigens) (European Commission Report, 1998). Cow's milk allergy (CMA) is an important problem in infants, and affects 1.9 to 2.8% of infants in the first two years of life in various countries of Northern Europe (Jacobsson and Lindberg, 1979; Host and Halken, 1990; Host et al., 1995; Host and Halken, 1998). Different proteins, i.e., β -lactoglobulin (BLG), casein, α -lactalbumin and serum albumin, have been identified as allergens of cow's milk (Wal, 1998), and sensitization to β LG has been demonstrated in more than 60% of CMA infants (Goldman et al., 1963; Lebenthal, 1975). Food allergy generally corresponds to an immediate type, IgE-mediated, immune response (Ford et al., 1983; Hill et al., 1989). This humoral response is considered to result from the activation of the Th2 type of T helper lymphocytes. In mice, the Th2 response results in IgE and IgG1 production, while the Th1 response leads to IgG2a production (Mosmann et al., 1986; Halstensen, 1997). Therefore, a high anti-BLG specific IgE responder mouse model is a useful tool for studying the mechanisms of induction and repression of allergy. Such a project requires the parallel monitoring and quantification of both total and allergen-specific IgE, IgG1 and IgG2a, in order to follow the Th1 and Th2 responses, respectively. The aim of this study therefore was to develop immunoassays that permit quantitative measurement of these different isotypes and subclasses.

The measurement of total Ig isotypes has been previously reported by different authors using two-site immunometric immunoassays (sandwich assay) (Kemeny et al., 1989; Larsson et al., 1997; Dearman et al., 1998). The capture step is ensured by anti-isotype Abs while another labeled anti-isotype Ab recognizing another epitope acts as the tracer. Quantification is performed using known concentrations of commercially available purified polyclonal Ig isotypes to plot standard curves. However, while the measurement of total isotype production is easy to perform, the quantification of allergen-specific antibodies is much more difficult. Two different strategies have been used. In the first, capture is ensured

by an anti-isotype specific antibody while paratope specificity is determined using a labeled antigen (Plebani et al., 1986; Hirano et al., 1989; Sakaguchi et al., 1989; Strouse et al., 1991; Olivieri et al., 1993). In the second, specific capture is ensured using a coated antigen and visualization is based on the use of an anti-isotype labeled antibody (Wal et al., 1995). These techniques suffer from several drawbacks. In the first case, the coated anti-isotype Ab binds the isotype irrespective of its paratopic specificity. Therefore, due to the limited binding capacity of the solid-phase, this method is dependent on the ratio of specific to total isotype concentration and probably underestimates specific antibody concentration (Plebani et al., 1986). The reverse situation is true for the second method, in which the coated allergen specifically immobilizes specific Abs whatever their isotype. However, because specific IgG are currently much more concentrated than the corresponding IgE (e.g., by at least a 3-log order of magnitude (Plebani et al., 1986)), they can compete with IgE for solid-phase binding, thus possibly leading to the underestimation of IgE levels. Moreover, whatever the method, the lack of a relevant standard (polyclonal allergen-specific IgE and IgG) makes absolute quantification difficult, leading at best to a semi-quantitative comparison of different sera.

In this paper, we describe two sets of enzyme immunoassays, suitable for quantitative measurement of total IgG1, IgG2a, and IgE on the one hand, and of BLG-specific IgG1, IgG2a and IgE on the other hand. The total Ig isotype assays use a classical method, i.e., two-site immunometric assays using two different anti-isotype Abs from commercial sources. Standard curves are established using commercially available purified Ig fractions. The choice of BLG as a model was based on two considerations. First, this protein is a major allergen from cow's milk, and is therefore relevant in establishing an animal model of allergy (Wal et al., 1995). Second, we have recently obtained a wide range set of mouse monoclonal antibodies (mAbs) which have been finely characterized in terms of isotype and specificity. Some of them have been used previously to develop a two-site immunometric assay suitable for measuring native and denatured forms of the protein (Négroni et al., 1998). These mAbs can be advantageously used as standards for allergen-specific iso-

type assays. We thus developed two-site immunometric assays using coated BLG as capture antigen, while anti-BLG isotypes (IgE, IgG1 and IgG2a) were quantified using the same enzymatic tracers previously used for total isotype determination. Standard curves were established using anti-BLG mAbs of different isotypes. Particular attention was paid to the method of coating the antigen on the solid phase, in order to optimize the binding of allergen-specific antibodies.

All these enzyme immunometric assays meet the sensitivity, reproducibility, isotype-specificity and quantification criteria. They were used to follow the humoral response of mice sensitized with alum-adsorbed BLG.

2. Materials and methods

2.1. Apparatus

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

2.2. Reagents and buffers

Unless otherwise stated, all reagents were of analytical grade and from Sigma (St Louis, MO). Bovine β -lactoglobulin (BLG) B variant was prepared as previously described (Wal et al., 1995). Anti-BLG specific IgG1 and IgG2a monoclonal antibodies (mAbs) were obtained as previously described (Négroni et al., 1998) and purified using either caprylic acid precipitation for IgG1 mAbs (Reik et al., 1987) or protein A (Bioprocessing, Princeton, NJ) affinity purification for IgG2a mAbs. These mAbs were previously characterized as directed against native BLG (BLG-1N, BLG-11N, BLG-18N, BLG-88N, BLG-97N, BLG-103N, BLG-117N) or preferentially reduced carboxymethylated («denatured») BLG (BLG-8R, BLG-45R, BLG-74R, BLG-92R). The BLG-specific IgE mAb was a gift from Dr Granato (Centre de Recherches Nestlé, Lausanne,

Switzerland). Rat anti-mouse IgE mAb (clone R-3592) and rat anti-mouse IgG2a mAb (clone R-1915) were from Pharmingen (San Diego, CA). Rat anti-mouse IgE mAb (clone LOME-3) was from Serotec (Oxford, England). Goat anti-mouse IgG1, goat anti-mouse IgG2a polyclonal affinity-purified antibodies as well as purified mouse IgGs, IgE, IgA and IgM were from Southern Biotechnology Associates (SBA, Birmingham, AL).

Buffers: (1) EIA buffer: 0.1 M phosphate buffer pH 7.4 containing 0.1% bovine serum albumin, 0.15 M NaCl, and 0.01% sodium azide; (2) Washing buffer: 0.01 M phosphate buffer pH 7.4 containing 0.05% Tween 20; (3) Ellman's medium: 7.5×10^{-4} M acetylthiocholine and 2.5×10^{-4} M DTNB in 0.1 M phosphate buffer pH 7.4.

2.3. Coating of microtiter plates

Direct coating of 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) was performed for 18 h at 20°C by reacting 120 μ l/well of either capture antibody (2.5 μ g/ml) diluted in 50 mM phosphate buffer pH 7.4 or BLG (5 μ g/ml) diluted in 25 mM Na₂EDTA, pH 9.3. After washing, plates were saturated with EIA buffer (300 μ l/well), sealed and stored at 4°C until use. When stored under these conditions, we did not observe any modification of their binding properties over at least a 2-month period.

Indirect BLG-coating was obtained through streptavidin–biotin interaction. Streptavidin was first coated on the plates by reacting 120 μ l/well of a 2.5 μ g/ml solution diluted in 50 mM phosphate buffer pH 7.4 for 18 h at 20°C. After washing, plates were saturated with 300 μ l/well of PVP buffer (0.1 M phosphate buffer pH 7.4, 0.4 M NaCl, 1% polyvinyl pyrrolidone, 1 mM EDTA and 0.01% sodium azide) for 18 h at 4°C. After washing, 125 μ l/well of a 5 μ g/ml of biotinylated-BLG solution (see below) in 0.1 M phosphate buffer was added to each well for a 3-h reaction at 20°C. Plates were extensively washed prior to use.

2.4. Biotinylation of BLG

Purified BLG (5 mg/ml in 50 mM NaHCO₃, pH 8.5) was reacted with different molar ratios (NHS-

LC-biotin/BLG; 2/1, 5/1, 20/1) of NHS-LC-biotin (Pierce, Rockford, IL) for 1 h at 4°C. Biotinylated-BLG was analyzed by mass spectrometry (MALDI-TOF MS) using a Voyager DE RP apparatus (PE Biosystems, Courtaboeuf, France).

2.5. Purification of acetylcholinesterase

Acetylcholinesterase (AChE) (EC 3.1.1.7) was extracted from the electric organ of the electric eel *Electrophorus electricus* and purified by one-step affinity chromatography, as described by Massoulié and Bon (1976). The tetrameric form of AChE (the polymorphism of AChE is detailed elsewhere (Massoulié and Bon, 1982)) used in this study and named “G4” was prepared from the purified AChE preparation by treatment with trypsin (1 µg/ml) for 18 h at 25°C in 0.1 M phosphate buffer pH 7 (1/2000 trypsin-AChE ratio (w/w)). Measurement of AChE activity using Ellman’s method were performed as previously described (Grassi et al., 1989).

2.6. Preparation of enzymatic tracers

F(ab')₂ fragments of goat anti-mouse IgG1 and IgG2a polyclonal antibodies, and rat anti-mouse IgE mAb (clone LOME-3) were obtained by treatment with pepsin in acidic medium (Lamoyi, 1986). These fragments were subsequently reduced to the Fab’ form in the presence of 2×10^{-2} M 2-mercaptoethylamine and covalently coupled to AChE pretreated with *N*-succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) as previously described (Grassi et al., 1989).

2.7. Enzyme immunometric assays for IgE, IgG1 and IgG2a

2.7.1. Measurement of total isotype and subclass concentrations

These quantifications were achieved using a conventional two-step procedure. Anti-isotype specific antibodies (anti-IgE: clone R35-92, anti-IgG2a: clone R19-15, and polyclonal anti-IgG1) coated on the plate ensured the capture of the Ig isotype present in the 100 µl volume (standard or diluted plasma sam-

ple, in duplicate) by reaction for 18 h at 20°C. Dilutions of the standards and of the samples (from 1/100 to 1/10⁷ depending on the assay and on the sample) were performed in EIA buffer. The plates were washed before addition of 100 µl of the corresponding anti-isotype-AChE conjugate (3 Ellman Units/ml; for the definition of the Ellman Unit, see Pradelles et al. (1985)) for a further 3-h reaction. Plates were then extensively washed and solid-phase bound AChE activity was determined by addition of 200 µl of Ellman medium. After 1 h incubation at room temperature, absorbance values were measured at 414 nm.

2.7.2. Measurement of anti-BLG IgE, IgG1 and IgG2a

Purified BLG was used as capture antigen, after being either passively adsorbed onto the microtiter plates or bound to coated streptavidin after biotinylation. The same procedure as described above for the measurement of total isotype concentration was performed. The standard curves were obtained using different anti-BLG mAbs, i.e., one IgE mAb, an equimolar mixture of 4 IgG1 mAbs (mAbs BLG-1N, BLG-8R, BLG-11N and BLG-74R) and an equimolar mixture of 2 IgG2a mAbs (mAbs BLG-88N and BLG-92R) were used for allergen-specific IgE, IgG1 and IgG2a assays, respectively. The last two pools involved mAbs recognizing different parts and forms (native or denatured) of BLG (Négroni et al., 1998). The IgG2a standard was composed of only 2 mAbs because antibodies of this subclass were scarce in the series of mAbs produced against BLG.

2.8. Efficiency of the capture step

To evaluate the capture efficiency in the different assays, standards were first reacted in plates coated either with antibodies or with BLG for 18 h at 20°C. The supernatants were then transferred into another similar plate (antibody or BLG-coated) for a second overnight reaction. The assays were further performed as described above for both series of plates. After measurement of solid phase-bound AChE activity, the ratio of the signals obtained after the second relative to the first incubation was calculated.

2.9. Specificity of the antibodies used as tracers

To test the specificity of each of the tracers used, murine standard Igs (IgE, IgG subclasses, IgA and IgM) were directly coated onto plates for 18 h at 20°C at different concentrations (ranging from 1 ng/ml to 10 µg/ml). After saturation with EIA buffer (see above), the AChE-labeled tracer antibodies (anti-IgE, anti-IgG1 or anti-IgG2a) were added for a 3-h reaction. The plates were then washed before Ellman medium was added for visualization.

2.10. Specificity of the assays

To test the specificity of each sandwich assay, IgE, IgG1 and IgG2a assays were performed as described above using murine standard Igs of each isotype (IgE, IgG subclasses, IgA and IgM) in concentrations ranging from 0.1 ng/ml to 10 µg/ml. Cross-reactivity (expressed as %) was arbitrarily defined as the ratio of concentration of the standard and of another isotype providing the same 0.5 AU signal.

2.11. Immunization of mice

Four-week-old female BALB/C mice from CERJ (Centre d'Élevage René Janvier, Le Genest St-Isle, France) were housed under standard animal hus-

bandry conditions, and were acclimatized for 2 weeks before immunization. The absence of BLG contamination in mouse feed was verified using a sensitive sandwich enzyme immunoassay developed in our laboratory (Négroni et al., 1998). Three mice were immunized i.p. with 100 µl of BLG (1 mg/ml) adsorbed on alum (Alhydrogel 3%, Superfos Biosector a/s, Denmark). The final volume for each immunization was 200 µl/mouse. The control group (3 mice) received an injection of alum alone. Mice were bled from the retro-orbital venous plexus before the first immunization and on days 11 and 18 post-immunization. After centrifugation and addition of sodium azide as preservative, sera were stored at –20°C until assay.

3. Results

3.1. Total IgG1, IgG2a and IgE sandwich assays

Standard curves established for total IgG1, IgG2a and IgE assays are shown in Fig. 1. The minimum detectable concentrations (MDCs, defined as the lowest concentrations of standard inducing a statistically significant increase in the absorbance measured for non-specific binding ($\text{mean} + 3\sigma_{n-1}$)), for total IgE, IgG1 and IgG2a assays were 4.9 pg/ml, 0.5 pg/ml and 43.3 pg/ml, respectively. The precision

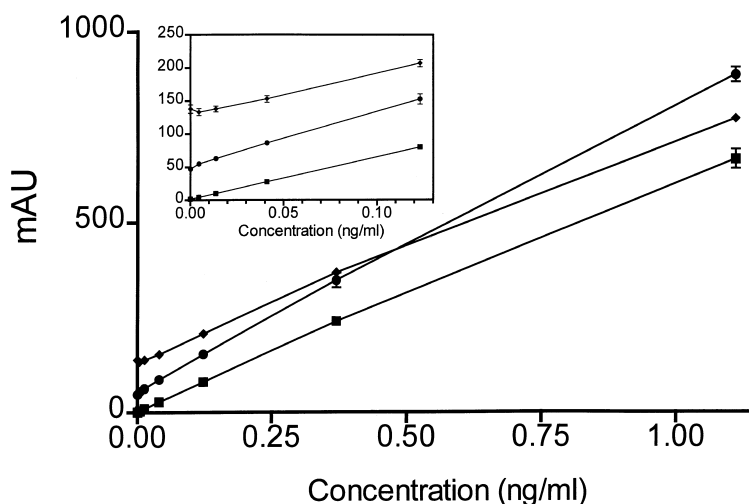


Fig. 1. Standard curves for total IgG1 (●), IgG2a (◆) and IgE (■). Each point represents the mean of quintuplicates $\pm$ S.D.

of these assays was also very satisfactory, with coefficients of variation less than 10% for the whole range of concentrations covered by the standard curves (data not shown). The inter-assay variation was also less than 10% (data not shown).

3.2. Sandwich immunoassays for the measurement of anti-BLG IgG1, IgG2a and IgE

3.2.1. Optimization of the BLG coating

Two different procedures for coating BLG were compared in order to optimize the capture of polyclonal antibodies contained in the antiserum of immunized animals. We used either a direct passive adsorption of the BLG onto the plates or an indirect coating procedure involving the binding of biotinylated BLG to streptavidin-coated plates. Different ratios of biotinylation were tested. As ascertained by mass spectrometry, reactions with 2-, 5- and 20-molar excesses of NHS-activated biotin led to a statistical incorporation in BLG of close to 1, 2 and 10 biotin molecules, respectively (data not shown).

Using several anti-BLG mAbs, which were previously demonstrated as directed against either the denatured form of BLG (e.g., recognizing “linear” epitopes), or the native form of BLG (e.g., binding to “conformational” epitopes), we compared the capture efficiency of these two different coating procedures. As shown in Fig. 2, monobiotinylated BLG

provided the best binding efficiency. Interestingly, derivatization with excess biotin appeared to reduce the recognition of BLG, especially for mAbs directed against native BLG.

When comparing passive adsorption and binding of monobiotinylated BLG (biotBLG), only small differences (less than 25% of the peak signal) were observed for the first set of mAbs (mAbs BLG-45R, BLG-74R and BLG-92R), both types of coating being correctly recognized, with a slight advantage for passive BLG adsorption. However, with conformational antibodies (BLG-1N, BLG-11N, BLG-18N, BLG-97N, BLG-103N, BLG-117N), direct coating gave a very small signal when compared with biotBLG immobilization. This demonstrates that direct adsorption of the protein on the polystyrene surface greatly alters the conformation of BLG. The use of multibiotBLG gave results close to passive adsorption and only the mAbs directed against linear epitopes recognized the coated antigen. In contrast, biotBLG coating appears to be a good compromise allowing recognition of the antigen by both types of mAbs. This coating procedure was then adopted for further experiments.

3.2.2. Standard curves for anti-BLG IgG1, IgG2a and IgE assays

As explained in the Methods section, we used anti-BLG mAbs of IgG1 (a mixture of 4 mAbs),

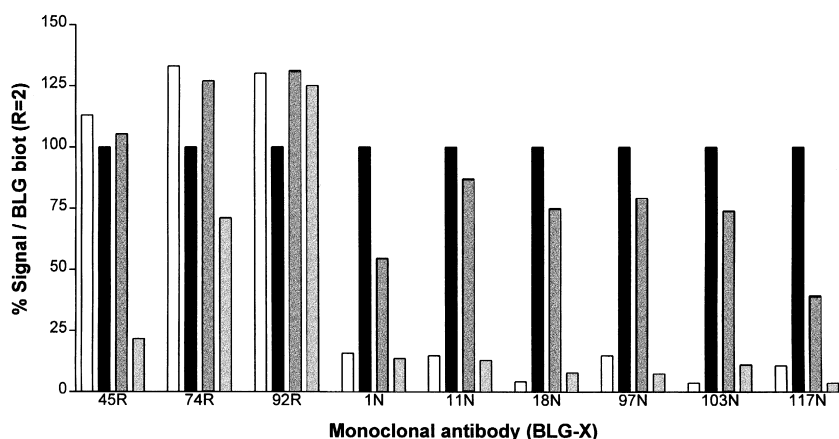


Fig. 2. Binding of monoclonal antibodies (mAbs) recognizing either denatured (mAbs BLG-45R, BLG-74R, BLG-92R) or native BLG (mAbs BLG-1N, BLG-11N, BLG-18N, BLG-97N, BLG-103N, BLG-117N) to either passively coated BLG (open bars) or biotBLG bound to streptavidin coated plates. Biotin: BLG molar ratio was 2 (full bars), 5 (dotted bar), or 20 (hallow bar). The signal obtained with a biotin: BLG molar ratio of 2 was used as the reference (100%).

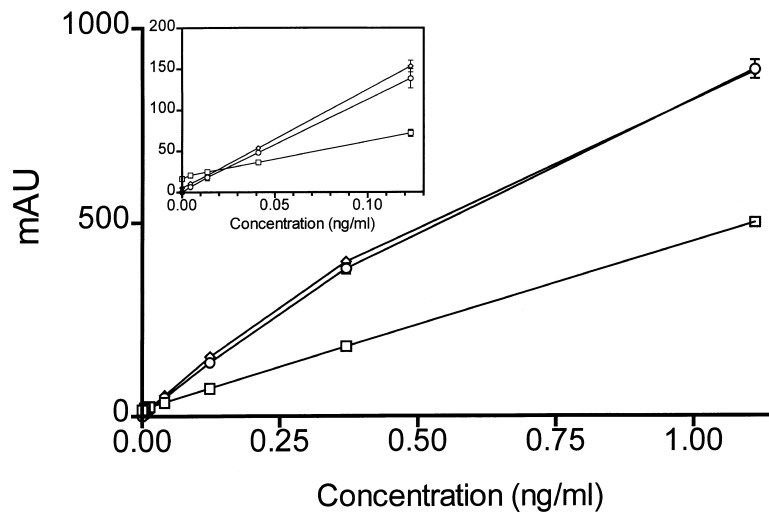


Fig. 3. Standard curves for specific antibodies IgG1 (○), IgG2a (◇) and IgE (□). Standard curves for specific antibodies were established using calibrated anti-BLG monoclonal antibodies, as described in the Material and Methods section. Each point represents the mean of quintuplicates $\pm$ S.D.

IgG2a (a mixture of 2 mAbs) and IgE (one mAb) isotypes as standard. These mAbs were calibrated using the total IgG1, IgG2a and IgE assays (see above). Using these mAbs, we obtained standard curves (Fig. 3) with MDC of 7, 12 and 8 pg/ml for anti-BLG IgG1, IgG2a and IgE assays, respectively. These assays have precision characteristics similar to those observed with total Ig assays, with coefficients of variation less than 10% within the concentration range used (data not shown). The inter-assay variations were also inferior to 10%. It is worth noting that the standard curves established for specific Ig assays were not strictly identical to those obtained for total Ig assays. This point will be discussed further.

3.3. Specificity of the assays

The specificity of the different total Ig assays was checked by comparing standard curves obtained with the target Ig to those obtained with other isotypes, i.e., IgE, IgG1, IgG2a, IgG2b, IgG3, IgA, IgM. All the assays proved to be specific, with less than 0.1% of cross-reactivity. For BLG-specific assays, it was not possible to perform a similar analysis since anti-BLG IgG2b, IgG3, IgA and IgM isotypes were not available. However, when using IgG1, IgG2a and IgE anti-BLG mAbs as standard with immobilized biotBLG, no cross-reactivity was observed with the heterologous anti-isotype tracers. In addition, we verified the specificity of the anti-isotype labeled

Table 1

Comparative measurement of the capture efficiency of anti-isotype or biot-BLG bound to streptavidin-coated plates. The percentage of antibodies present in the supernatant of a first capture reaction was determined by performing a second capture in the same conditions. Binding observed in the second capture reaction is expressed as a percentage of the binding observed during the first capture reaction

Samples	Total isotype			Specific isotype		
	Coating: Anti-IgE Anti-IgG1 Anti-IgG2a			BLG-biot		
	IgE	IgG1	IgG2a	IgE	IgG1	IgG2a
Standard (purified isotypes commercially available, 1 ng/ml in EIA buffer)	0	0.8	5.1			
mAb (1 ng/ml) in EIA buffer				35	16	12
Standard or mAb (1 ng/ml) in non-immune sera	4.9	1.7	16	34	15	10

antibodies using serial dilution of the different purified isotypes, i.e., IgE, IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, directly coated onto plates. Under these conditions, we determined the cross-reactivities to be less than 0.1% for the three different conjugates for the nonrelevant coated isotypes (data not shown). These assays thus allow unambiguous detection and quantification of all specific anti-BLG isotypes, even IgE antibodies which are present at a much lower concentration than are the IgG antibodies.

Non-specific binding of Ig was also evaluated by assaying mice sera on BSA-, HSA-, KLH- and casein-coated plates, providing results equivalent to non specific binding of the tracer antibodies (data not shown). This further demonstrates that the specific antibodies detected are BLG-specific antibodies.

3.4. Efficiency of the capture step

As previously reported (Grassi et al., 1986), the quantification of antibodies is based on the hypothesis that the capture of Ig is total or equivalent for the standard antibodies and the serum antibodies. This was checked by analyzing the supernatant of an 18-h capture reaction of the samples (standard or mAbs) with the two different solid-phase (anti-isotype antibodies or biotBLG) after transfer to another similar solid-phase (see Section 2). As shown in Table 1, the residual concentration of IgGs in the first supernatant was less than 16%, and currently less than 10%, demonstrating that the capture efficiency (ranging from 99 to 84%) was fairly similar for the different total or anti-BLG isotype immunoassays. For specific IgE, the capture efficiency was lower (mAb diluted in EIA and in serum), but was equivalent in the two cases.

3.5. Parallelism of standard and serum dilution curves:

As shown in Fig. 4, serial dilutions of antisera were parallel to the standard curves (established with anti-BLG mAbs), suggesting that the use of anti-BLG mAbs as standards was representative of the polyclonal response. The same parallelism was also observed in the case of total Ig assays (data not shown).

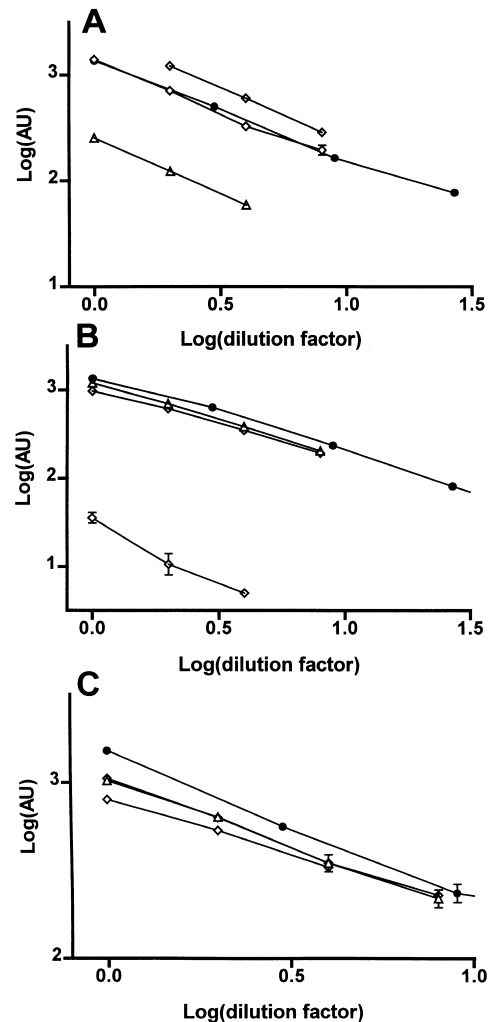


Fig. 4. Measurement of anti-BLG IgG1 (A), IgG2a (B) and IgE (C): parallelism between the standard curves established with mAbs (—•—) and serum dilution 11 days (—△—) or 18 days (—◇—) after immunization, on biotBLG bound to streptavidin coated plates. Values used as a reference are: serum dilution = $1/10^5$, $1/10^3$ and $1/50$ for IgG1, IgG2a and IgE, respectively; standard concentration = 0.1, 0.3 and 3 ng/ml for mAbs IgG1, IgG2a and IgE. Each point represent the mean value $\pm$ SD of duplicate determinations.

3.6. Analysis of the humoral response in mice sensitized with BLG.

Total and anti-BLG Ig concentrations were thus measured in the sera from three mice immunized with BLG adsorbed on alum. As a control, alum was

administered alone (Fig. 5, open symbols). In control mice, an increase in the IgG2a response, reaching 750 $\mu\text{g}/\text{ml}$ on day 18, was observed, corresponding to a 10-fold increase compared to the concentration measured on day 0. No change was observed in the IgG1 and IgE responses, with respective mean values of about 500 $\mu\text{g}/\text{ml}$ and 100 ng/ml.

When administering 100 μg of BLG adsorbed on alum, important changes were seen in total IgG2a,

IgG1 and IgE responses (Fig. 5, filled squares). Total IgG2a was reduced on day 18 with respect to controls, while total IgG1 and total IgE were increased. On day 18, IgG1, IgE and IgG2a reached values close to 600 $\mu\text{g}/\text{ml}$, 570 ng/ml, and 410 $\mu\text{g}/\text{ml}$, respectively. Variability was notable and depended on the mice, suggesting a variability in the specific response. This specific response was essentially of a Th2-type, with a concomitant increase in specific

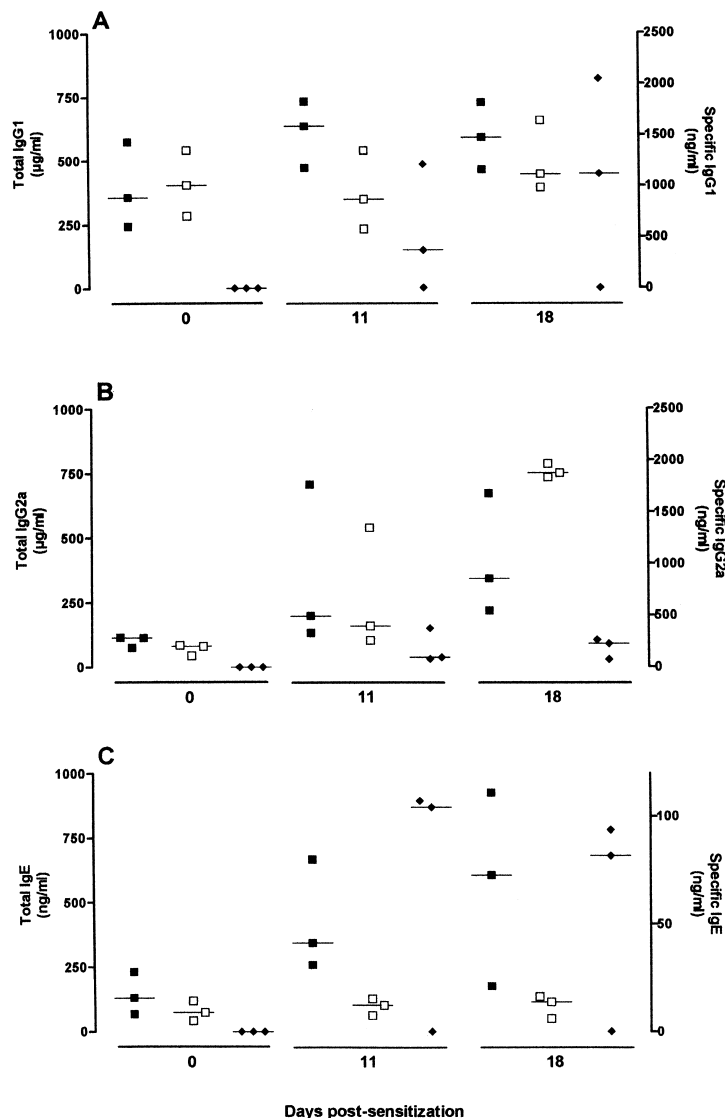


Fig. 5. Humoral response in mice immunized with 100 μg of BLG adsorbed on alum. Total Igs (■) and BLG-specific Igs (◆) are represented: IgG1 (A), IgG2a (B) and IgE (C) are shown. Each point represents an individual mouse. □ indicates control mice (immunized with the adjuvant alone), and the median is represented by a line. No BLG-specific antibodies were detected in the control mice.

IgE and IgG1, and a low IgG2a-specific response. One mouse was not sensitized to BLG and gave a very low specific response: no IgE were detectable, and only 0.6 ng/ml and 70 ng/ml of specific IgG1 and IgG2a, respectively, were measurable. In the two other mice, specific IgG2a, IgG1 and IgE reached levels of 240 ng/ml, 1500 ng/ml and 90 ng/ml, respectively, on day 18. It is interesting to note that specific IgG1 and IgG2a represented only a minor part of the total IgG (about 0.15 and 0.35%, respectively), whereas specific IgE represented about 12% of the total IgE response.

Since specific IgG levels are higher than specific IgE levels, we have verified the absence of interference for BLG binding. Spiking the day 0, day 11 and day 18 sera of a representative mouse with mAb anti-BLG IgE at doses from 800 to 25 pg/ml provided good results. Recovery of more than 80% of anti-BLG IgE, naturally induced plus added standards, was always reached. This suggests the absence of any major interference of IgG on IgE binding by competition during the primary response.

4. Discussion

In this paper, we describe immunoassays which permit the quantification of both total and BLG-specific IgE, IgG1 and IgG2a. We have shown that these assays are specific, very sensitive (MDC close to 10 pg/ml), and fulfill the various criteria required for validation (capture efficiency of the different types of coated solid-phase, parallelism between standard curves and dilution of sera). Previous publications have reported immunoassays to quantify total serum Ig isotypes (Kemeny et al., 1989; Larsson et al., 1997; Dearman et al., 1998). These methods have similar specificity to the assays described here. However, to the best of our knowledge, this is the first description of immunoassays allowing quantification of antigen-specific Ig isotypes and their comparison with total isotype concentrations. This was possible in the case of BLG because of the availability of different mAbs directed against this protein (Négroni et al., 1998). This allowed us to prepare mixtures of mAbs recognizing both the native and denatured forms of the allergen, thus mimicking the polyclonal response occurring *in vivo*. Their use

provided a way to achieve a satisfactory quantitative analysis of the Ig produced by immunized animals. Allergen-specific Ig concentration measurements have been reported in several studies, but in most cases only standard curves for total IgX using a classical sandwich assay were generated and specific IgX concentrations were extrapolated from these standard curves. These methods permit only semi-quantitative measurements and currently overestimate specific Ig concentrations. We experienced the same problem in preliminary experiments based on this approach. For instance, we observed that specific IgG1 concentration estimated from the total IgG1 standard curve could represent up to 100 to 150% of total IgG1 (data not shown). It seems that in the total Ig assays, a fraction of the immobilized Ig is unavailable for tracer binding, possibly due to steric hindrance not encountered when Ig are bound to the solid-phase by their paratope. The difference in accessibility in the total and specific assays can be partly circumvented by using a combination of anti-Fab and anti-Fc antibodies for the capture and visualization steps, respectively, but the use of a single total Ig standard curve will not guarantee correct quantification (Gupta and Siber, 1995). The availability of anti-BLG specific mAbs and their use as the standards enabled us to avoid this problem and perform totally calibrated assays for both total and antigen-specific assays, since mAbs and serum Ig are recognized in the same way by tracer Abs. The only parameter which differs between these two assays (total and specific) is the use of different coated solid-phases, either anti-isotype Ab or the biotinylated antigen. In all other aspects the processes are strictly identical. As a consequence, comparison of these two assay systems requires verification of similar capture efficiencies and of parallelism between the dilutions of the sera and the standard curves. These two criteria were clearly satisfied in the present assays.

BLG-specific Igs were detected by using indirect coating of the antigen. Direct coating of an antigen has already been described as partly denaturing (Wal et al., 1995; Selo et al., 1996). This was illustrated by the lack of recognition of passively coated BLG by anti-BLG mAbs directed against «conformational» epitopes, resulting from possible masking and/or partial denaturation of epitopes. Using bio-

tinylated BLG bound to streptavidin, we confirmed that the biotin incorporation rate is an important factor that must be controlled. Incorporation of more than a single biotin molecule results in decreased recognition of BLG by mAbs, particularly anti-conformational ones. In contrast, we found that use of a monobiotinylated derivative of BLG associated with streptavidin-coated plates permitted efficient binding of both types of mAbs, i.e., directed against native as well as denatured BLG. This point is critical since the different adjuvants known to induce a high IgE response (Alum, as used in the present study, Complete/Incomplete Freund's Adjuvant, *Pertussis* toxin) have been previously described as favoring the production of antibodies raised against different structural features of the protein, alum mainly presenting a non denatured form of the antigen, unlike CFA (Claassen and Boersma, 1992). The development of a good high-IgE responder model implies comparison of these adjuvants, and thus requires immunoassays allowing the detection of antibodies directed against both types of epitope ("linear" and "conformational"). This type of comparison (adjuvant and dose effect) is currently under investigation in our laboratory, and is being analyzed using these new quantitative assays.

As an illustration of the potential interest of these assays, we analyzed the humoral response in mice sensitized with 100 µg of BLG adsorbed on alum. We compared the production of total and specific antibodies using our quantitative assays. This allowed us to determine the concomitant production of specific IgE and IgG1, representative of the Th2 response, with low levels of specific IgG2a. The specific IgE serum content peaked at 100 ng/ml for two mice on day 11, and was maintained until day 18. By studying the primary response, we have observed that specific IgG isotypes are 10 to 20 times more concentrated than specific IgE. However, we have shown that this excess did not induce any interference, i.e., by competition of the different isotypes for coated BLG, in specific IgE assays. This could be completely different after a secondary immune response, i.e., after a booster injection, which results in production of high levels of the IgG isotypes. In the present study, the proportion of specific vs. total IgE was about 12%, whereas it was less than 0.5% for IgG1 and IgG2a. This high specific

IgE level demonstrates the possibility of using BLG to develop an animal model of allergy, since BLG is known to be a potent allergen (Wal et al., 1995).

In conclusion, quantitative assays for total and allergen-specific IgE, IgG1 and IgG2a proved to be useful tools when studying the humoral response of mice after immunization. They will facilitate comparisons between different adjuvants and allergen dose for optimizing the IgE production during the development of a high responder IgE mice model. Other parameters can be studied, such as the balance between specific IgE and IgX, and the relative proportion of specific vs. total IgX, or the IgG1/IgG2a ratio (total as well as specific) reflecting the Th1 or Th2 orientation of the response. Finally, these assays should be considered for the evaluation of different immunotherapy protocols used to treat allergy in mice.

References

- Claassen, E., Boersma, W.J.A., 1992. Characteristics and practical use of new-generation adjuvants as an acceptable alternative to Freund's complete adjuvant. *Res. Immunol.* 143, 475–477.
- Dearman, R.J., Basketter, D.A., Blaikie, L., Clark, E.D., Hilton, J., House, R.V., Ladics, G.S., Loveless, S.E., Mattis, C., Sailstad, D.M., Sarlo, K., Selgrade, M.K., Kimber, I., 1998. The mouse IgE test: interlaboratory evaluation and comparison of BALB/c and C57BL/6 strain mice. *Toxicol. Methods* 8, 69–85.
- European Commission Report, Task 7. 2. Scientific co-operation on questions relating to food: consideration of the epidemiological basis for appropriate measures for the protection of public health in respect to food allergy. European Commission Report, Task 7.2. 26-5-1998.
- Ford, R.P.K., Hill, D.J., Hosking, C.S., 1983. Cow's milk hypersensitivity: immediate and delayed onset clinical patterns. *Arch. Dis. Child.* 58, 856–862.
- Goldman, A.S., Anderson, D.W., Sellers, W.A., Saperstein, S., Kniker, W.T., Halpern, S.R., 1963. Milk allergy: I. Oral challenge with milk and isolated milk proteins in allergic children. *Pediatrics*, 425–443.
- Grassi, J., Didierlaurent, A., Stadler, B.M., 1986. Quantitative determination of total and specific human IgE with the use of monoclonal antibodies. *J. Allergy Clin. Immunol.* 77, 808–822.
- Grassi, J., Frobert, Y., Pradelles, P., Chercuitte, F., Gruaz, D., Dayer, J.-M., Poubelle, P.E., 1989. Production of monoclonal antibodies against interleukin-1 α and -1 β . Development of two enzyme immunometric assays (EIA) using acetylcholinesterase and their application to biological media. *J. Immunol. Methods* 123, 193–210.
- Gupta, R.K., Siber, G.R., 1995. Method for quantitation of IgG

- subclass antibodies in mouse serum by enzyme-linked immunosorbent assay. *J. Immunol. Methods* 181, 75–81.
- Halstensen, S.T., 1997. Why are we not all allergic: basic mechanisms for tolerance development. *Environ. Toxicol. Pharmacol.* 4, 25–31.
- Hill, D.J., Firer, M.A., Shelton, M.S., Hosking, M.D., 1989. Manifestations of milk allergy in infancy: clinical and immunologic findings. *J. Pediatr.* 109, 270–279.
- Hirano, T., Yamakawa, N., Miyajima, H., Maeda, K., Takai, S., Ueda, A., Taniguchi, O., Hashimoto, H., Hirose, S., Okumura, K. et al., 1989. An improved method for the detection of IgE antibody of defined specificity by ELISA using rat monoclonal anti-IgE antibody. *J. Immunol. Methods* 119, 145–150.
- Host, A., Halken, S., 1998. Epidemiology and prevention of cow's milk allergy. *Allergy* 53, 111–113.
- Host, A., Halken, S.A., 1990. A prospective study of cow's milk allergy in Danish infants during the first 3 years of life. Clinical course in relation to clinical and immunological type of hypersensitivity reaction. *Allergy* 45, 587–596.
- Host, A., Jacobsen, H.P., Halken, S., Holmenlund, D., 1995. The natural history of cow's milk protein allergy/intolerance. *Eur. J. Clin. Nutr.* 49, S13–S18, Suppl. 1.
- Jacobsson, I., Lindberg, T.A., 1979. A prospective study of cow's milk intolerance in Swedish infants. *Acta Pediatr. Scand.* 68, 853–859.
- Kemeny, D.M., Richards, D., Durmin, S., Johannsson, A., 1989. Ultrasensitive enzyme-linked immunosorbent assay (ELISA) for the detection of picogram quantities of IgE. *J. Immunol. Methods* 120, 251–258.
- Lamoyi, E., 1986. Preparation of F(ab')₂ fragments from mouse IgG of various subclasses. *Methods Enzymol.* 121, 652–669.
- Larsson, P.H., Eklund, A., Johansson, S.G., Larsson, K., 1997. Covalent binding of proteins to grafted plastic surfaces suitable for immunoassays: II. Picograms of IgE detected in BAL fluid in sarcoidosis. *J. Immunol. Methods* 210, 41–49.
- Lebenthal, E., 1975. Cow's milk protein allergy. *Pediatr. Clin. N. Am.* 22, 827–833.
- Massoulié, J., Bon, S., 1976. Affinity chromatography of acetylcholinesterase. The importance of hydrophobic interactions. *Eur. J. Biochem.* 68, 531–539.
- Massoulié, J., Bon, S., 1982. The molecular forms of acetylcholinesterase in vertebrates. *Annu. Rev. Neurosci.* 5, 57–106.
- Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L., 1986. Two types of murine helper T cell clone: I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136, 2348–2357.
- Négroni, L., Bernard, H., Clément, G., Chatel, J.-M., Brune, P., Frobert, Y., Wal, J.-M., Grassi, J., 1998. Two-site enzyme immunoassays for determination of native and denatured β -lactoglobulin. *J. Immunol. Methods* 220, 25–37.
- Olivieri, V., Beccarini, I., Gallucci, G., Romano, T., Santoro, F., 1993. Capture assay for specific IgE. An improved quantitative method. *J. Immunol. Methods* 157, 65–72.
- Plebani, A., Ugazio, A.G., Avanzini, A.M., Monafò, V., Burgio, G.R., 1986. An enzyme-linked immunosorbent assay for cow's milk protein-specific IgE using biotinylated antigen. Avoidance of interference by specific IgG. *J. Immunol. Methods* 90, 241–246.
- Pradelles, P., Grassi, J., Maclouf, J., 1985. Enzyme immunoassay of eicosanoids using acetylcholine esterase as label: an alternative to radioimmunoassay. *Anal. Chem.* 57, 1170–1173.
- Reik, L.M., Maines, S.L., Ryan, D.E., Levin, W., Bandiera, S., Thomas, P.E., 1987. A simple, non-chromatographic purification procedure for monoclonal antibodies. Isolation of monoclonal antibodies against cytochrome P450 isozymes. *J. Immunol. Methods* 100, 123–130.
- Sakaguchi, M., Inouye, S., Miyazawa, H., Tamura, S., 1989. Measurement of antigen-specific mouse IgE by a fluorometric reverse (IgE-capture) ELISA. *J. Immunol. Methods* 116, 181–187.
- Selo, I., Negroni, L., Creminon, C., Grassi, J., Wal, J.M., 1996. Preferential labeling of alpha-amino N-terminal groups in peptides by biotin: application to the detection of specific anti-peptide antibodies by enzyme immunoassays. *J. Immunol. Methods* 199, 127–138.
- Strouse, R.J., Anderson, D.W., Argentieri, D.C., 1991. Rapid detection of antigen-specific mouse IgE by enzyme-linked immunosorbent assay (ELISA). *J. Immunoassay* 12, 113–124.
- Wal, J.-M., Bernard, H., Yvon, M., Peltre, G., David, B., Créminon, C., Frobert, Y., Grassi, J., 1995. Enzyme immunoassay of specific human IgE to purified cow's milk allergens. *Food Agr. Immunol.* 7, 175–187.
- Wal, J.-M., 1998. Cow's milk allergens. *Allergy* 53, 1013–1022.