

Genetic Immunisation with Bovine β -Lactoglobulin cDNA Induces a Preventive and Persistent Inhibition of Specific Anti-BLG IgE Response in Mice

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Key Words

Allergy · DNA immunisation · Th1/Th2 · β -Lactoglobulin, bovine

Abstract

Background: Various studies have shown that DNA immunisation with gene allergen induces a non-allergic response. **Methods:** We applied this new type of vaccination to bovine β -lactoglobulin (BLG), a major cow's milk allergen, using a plasmid that allows the production of a partially secreted protein. Specific antibodies and cytokines were quantified in different immunisation protocols. **Results:** The primary response in mice immunised with BLG-encoding plasmid (pBLG) is of the Th1 type. Restricted recognition of a native form of BLG in pBLG mice contrasted with a broader range of recognition in BLG-in-alum-immunised mice, notwithstanding the fact that alum favours the presentation of a native form of the antigen. We also demonstrated an inhibitory effect of pDNA immunisation on the Th2 response induced by a subsequent immunisation using BLG adsorbed on alum. However, this preventive effect is highly dependent on the time of pre-administration of the pBLG, with an optimal effect when pDNA immunisation occurred at least 21 days before protein administration. This preventive ef-

fect resulted concomitantly in the inhibition of BLG-specific IgE, in the induction of specific IgG2a, and in the decrease of the specific IgG1/IgG2 ratio. It is accompanied by an increase in IFN γ and IL-10 secretion. Moreover, the preventive effect was shown to be persistent even after a booster immunisation with alum-adsorbed BLG. The Th1 orientation of the response is very likely due to the presentation of the protein in the Th1 environment due to plasmid immunostimulatory sequences, as intramuscular injection of BLG itself leads to a weak Th2 response and had no preventive effect on a subsequent sensitisation. **Conclusion:** This study further demonstrates the potential use of DNA immunisation for prevention of IgE response, but the window of action seems to be very restricted if we are to inhibit an established Th2 response efficiently.

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Introduction

Three to 5% of the population of western countries present immunologically mediated reactions against dietary antigens, i.e. food allergy. Cow's milk allergy affects 1.9–2.8% of young children [1], who may be sensitised to various proteins, mainly β -lactoglobulin (BLG) and ca-

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sein [2]. For instance, more than 60% of infants with cow's milk allergy have specific IgE against BLG [3, 4]. Food allergy corresponds essentially to an immediate-type, IgE-mediated, immune response [5–8], considered to result from activation of the Th2 type of T helper lymphocytes. In mice, this Th2 response is mainly characterised by the synthesis of interleukins (IL)-4, IL-5 and IL-13 which induce the production of IgE and IgG1. The Th1 response is characterised by IFN γ and IL-12 synthesis, and promotes an immunoglobulin class switch to IgG2a. The Th1 and Th2 responses mutually inhibit each other's development and function via the cytokines produced [9, 10]. It is therefore of interest to develop an immunotherapy allowing the induction of a Th1 response against allergens, without the risk of inducing anaphylaxis.

DNA immunisation consists on the administration of deoxynucleotide sequences encoding the target antigen inserted into a mammalian expression vector derived from prokaryote plasmids (plasmid DNA, pDNA). In 1990, intramuscular (i.m.) injection of such a plasmid in mice was shown to lead to the *in vivo* production of the encoded protein [11]. Two years later, it was demonstrated that this production allowed the induction of an immune response [12]. A number of publications subsequently showed that i.m. or intradermal (i.d.) administration of pDNA induces a Th1-type response. Such a polarisation of the immune response is thought to be due to the presence of immunostimulatory sequences (ISS) in bacterial DNA. These ISS consist of centred unmethylated CpG flanked by two 5' purines and two 3' pyrimidines [13]. Such sequences are more frequent in lower organisms than in vertebrates and are methylated in the latter. *In vitro* and *in vivo* studies have demonstrated that unmethylated CpG induce an innate response with production of IFN α/β , and IL-12 by APC, IL-6 and IL-12 by B cells. This cytokine environment leads to the production of IFN γ by NK cells and then to the induction of Th1 cells [13–17]. Codelivered antigen (for example after DNA immunisation) can then be presented to the immune system in a Th1 environment and induce an antigen-specific Th1 response. DNA immunisation has been successfully applied to allergens in order to induce a Th1 response specific to β -galactosidase [18, 19], ovalbumin [20], Der p 5 [21, 22], Hev b 5 [23], Ara h 2 [24], Bet v 1 [25], Cry j 1 [26]. DNA immunisation also effectively prevented an IgE response [18, 19, 21, 22, 24] and downregulated a pre-existing IgE response [18, 23].

The aim of our study was to apply DNA immunisation to BLG, a major cow's milk allergen. BLG is an 18-kD globular whey protein of the lipocalin family. It contains

two intramolecular S-S bridges and 1 free cysteine. Its 3-dimensional structure is well known. In a previous paper, we demonstrated that an i.m. injection of a plasmid encoding the full sequence of BLG allows production of the protein strictly in a native conformation at the injection site [27]. It has now been demonstrated that the primary immune response in mice immunised with BLG-encoding plasmid (pBLG mice) is of the Th1 type. Moreover, specific antibody response in pBLG mice was shown to be restricted to the native form of BLG, whereas immunisation with BLG in alum led to antibodies against native, denatured BLG (without intramolecular S-S bridges), and also against some BLG-derived peptides. A preventive effect of pBLG immunisation on the development of a Th2 response induced by a later intraperitoneal (i.p.) injection of BLG in alum was then evaluated, and we demonstrated that an appropriate latent period between a single i.m. DNA immunisation and i.p. protein sensitisation is determinant for the preventive effect. A maximal preventive effect was obtained with a latent period of 21 days, and was accompanied by nearly total inhibition of specific IgE, and marked induction of specific IgG2a. Secretion of IFN γ and IL-10 by spleen cells of pBLG pre-immunised mice increased with the length of the latent period. When a latent period of 21 days was applied, we further demonstrated that the effect of DNA immunisation persists after a booster BLG-in-alum injection, as proved by IgE inhibition and IgG1/IgG2a ratio. Epitopic recognition of specific IgG1 was greater when compared to the primary response after pBLG immunisation, unlike IgG2a recognition.

These results further demonstrated the single DNA immunisation potential for treatment of allergic diseases, but the window of action seems to be very restricted if we are to inhibit an established Th2 response efficiently.

Material and Methods

Apparatus

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

Antibodies, Reagents and Buffers

Unless otherwise stated, all reagents were of analytical grade from Sigma (St. Louis, Mo., USA).

Antibodies, Reagents and Buffer for Immunoassays. Antibodies and buffers used for determination of specific IgG1, IgG2a and IgE have been described in detail elsewhere [28].

BLG and BLG-Derived Products. BLG variant A and reduced and carboxymethylated BLG were prepared as previously described [29].

BLG synthetic peptides used were deduced from the sequence of tryptic peptides already recognised as allergenic epitopes [30] and synthesised using a Milligen 9050 apparatus and standard Fmoc chemistry. Peptides were purified by HPLC, checked by amino acid composition analysis and MALDI-TOF mass spectrometry using a Voyager DE RP apparatus (PE Biosystems, Courtaboeuf, France).

Construction of pcDNA-BLG Plasmid

The construction of the pcDNA-BLG plasmid (pBLG) has been described elsewhere [27]. Briefly, BLG cDNA from BLG variant A [kindly provided by C.A. Batt, 31] was PCR-amplified, with the addition of BLG peptide signal and Kozak consensus translation codon, deduced from the BLG full sequence [32]. The PCR product was ligated in two steps into pcDNA.3 plasmid (Invitrogen, The Netherlands) using *Hind* III and *Xba* I restriction enzymes. pcDNA.3 plasmid contains CMV promoter, bovine growth hormone polyadenylation signal and the ampicillin resistance gene. Plasmids were grown in DH5 α strains of *Escherichia coli*, and purified using Mega-Prep Endotoxin Free kit from Qiagen (Courtaboeuf, France). The quality and quantity of the plasmids were analysed by absorption at 260 and 280 nm and by agarose gel electrophoresis. Plasmids were stored at -20°C in 10 mM Tris HCl/0.1 mM EDTA, pH 8. Before administration, they were precipitated in 100% ethanol, washed with 70% ethanol, and dissolved in pyrogen-free PBS, at 2 mg/ml. In vitro and in vivo production of BLG has been quantified [27], showing a possible secretion of the protein. pcDNA.3 was used as plasmid control.

Immunisation of Mice

Female BALB/c mice were from CERJ (Centre d'Élevage René Janvier, Le Genest-St-Isle, France), and were housed under normal husbandry conditions. Mice were acclimated for 2 weeks before immunisation, and were used when 6–8 weeks old. The absence of BLG from mice feed was verified using a sensitive sandwich immunoassay [33].

Plasmid injections were performed i.m. in the tibialis anterior muscle of mice anaesthetised by an i.p. injection of sodium pentobarbital (75 mg/kg). Injections of 25% sucrose and 30 min later of 50 μl of the plasmid solutions (2 mg/ml) were performed using 27-gauge needles. The control group was immunised under the same conditions with 100 ng of BLG, 50 μl of PBS or 100 μg of control plasmid pcDNA.3 instead of pBLG. i.p. protein injections consisted of 200 μl /mouse of 50 μg of BLG, adsorbed on 1 mg of alum (Alhydrogel 3%, Superfos Biosector als, Detroit, Mich., USA). Blood was collected from the retro-orbital venous plexus and centrifuged, 0.1% sodium azide was added as preservative, and the samples were stored at -20°C until further assay.

Cytokine Productions

Spleens were harvested under sterile conditions. After lysis of red blood cells (180 mM NH_4Cl , 17 mM Na_2EDTA) and several washes, splenocytes were resuspended in RPMI 10 (RPMI supplemented with 10% FCS, 2 mM *L*-glutamine, 100 U penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin). Cells were incubated in 96-well culture plates (10⁶ cells/well) in the presence of BLG, or PBS or ovalbumin (25 $\mu\text{g}/\text{ml}$; negative control), or concanavalin A (1 $\mu\text{g}/\text{ml}$; positive control) for 60 h at 37°C (5% CO_2). Supernatants were then removed and stored at -80°C until further assay.

IL-4 and IFN γ were assayed using CytoSetsTM kits (BioSource International Europe, Nivelles, Belgium) according to the manufac-

turer's instructions. IL-5 was assayed using an immunometric assay described elsewhere [34], using monoclonal antibodies TRFK4 for capture and acetylcholinesterase (AChE)-labelled TRFK5 monoclonal antibodies for development. IL-10 was quantified by 'Solid-Phase Immobilised-Epitope Immunometric Assay' [35]. This protocol involves the following sequential steps: (1) immunocapture of IL-10 present in 100 μl of sample (or standard) by monoclonal anti-IL-10 antibodies JES 2A5-coated on microtiter plates, (2) cross-linking of IL-10 via its amino group to the solid phase using 2.5% glutaraldehyde diluted in 0.1 M phosphate buffer (10 $\mu\text{l}/\text{well}$), (3) treatment with 2 N HCl/methanol in borane, (4) after a washing step, measurement of linked IL-10 using the same monoclonal anti-IL-10 antibodies labelled with AChE. The detection limit of this assay is 30 pg/ml of culture media.

Quantification of BLG-Specific IgE, IgG1 and IgG2a

These assays are described in detail elsewhere [28]. Briefly, biotinylated BLG (5 $\mu\text{g}/\text{ml}$ in 0.1 M phosphate buffer pH 7.4) is immobilised on streptavidin-coated plates (5 $\mu\text{g}/\text{ml}$, 120 $\mu\text{l}/\text{well}$ in 50 mM phosphate buffer pH 7.4). This coating allows the detection of antibodies recognising both denatured and native forms of BLG. After an 18-hour incubation with standards and sera at convenient dilution and a wash step, 100 μl of AChE-labelled Fab' fragments of anti-mouse IgE (clone LOME-3, Serotec, Oxford, UK), anti-IgG1 or anti-IgG2a (Southern Biotechnology Associated, Birmingham, Ala., USA) were added for 3 h at 20°C . The plates were then washed before addition of 200 μl of Ellman's medium, allowing staining of solid phase-bound AChE at 414 nm. Quantification of specific IgE is preceded by removal of serum IgG using protein G immobilised on porous glass (PROSEP, Bioprocessing, Consett, UK), avoiding IgG interference in IgE detection. Specific IgE concentrations are corrected using a loss factor r ($r = \text{total IgE before protein G treatment} / \text{total IgE after protein G treatment}$), allowing calculation of actual concentrations.

Epitopic Specificity

Epitope specificity was assayed by analysing the binding of BLG, rcmBLG and BLG-derived peptides labelled with AChE to serum antibodies immobilised on anti-isotype-coated plates [anti-mouse IgE clone R-3592, anti-mouse IgG2a clone R-1915 (Pharmingen, San Diego, Calif., USA), or anti-mouse IgG1 (Southern Biotechnology Associated, Birmingham, Ala., USA)]. These different tracers were obtained by reaction of thiol groups previously introduced in the peptides with maleimido groups incorporated into AChE, as previously described [36].

Statistical Analysis

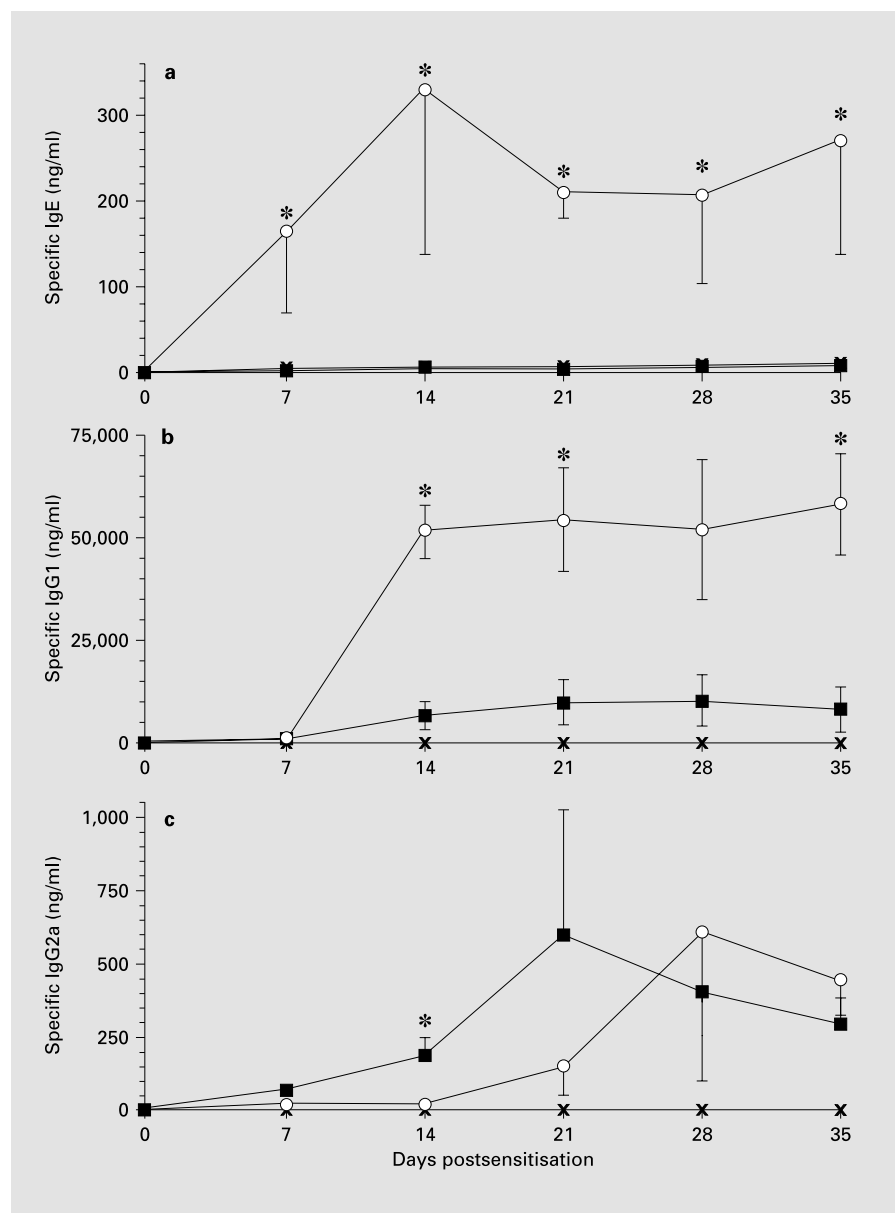
Statistical significance was analysed using Student's *t* test.

Results

Induction of a Th1-Type Immune Primary Response in Genetically Immunised Mice

BLG-Specific IgE, IgG1 and IgG2a in Protein or cDNA-Immunised Mice. In a first set of experiments, we analysed the primary immune response in mice immunised either i.p. with BLG in alum (alum-BLG mice) or i.m.

Fig. 1. Primary immune response after DNA immunisation: BLG-specific IgE (**a**), IgG1 (**b**) and IgG2a (**c**) were quantified in mice immunised on day 0 with 100 µg of pBLG (i.m.; ■), 100 ng of BLG (i.m.; ×), or 50 µg of BLG adsorbed on alum (i.p.; ○). Mean ± SD of 5 mice per group are represented. No specific antibodies were detectable in mice immunised with control plasmid (not shown). Significant differences between pBLG and BLG-in-alum-immunised mice are indicated (* $p < 0.05$, t test).



with BLG (BLG mice), with a plasmid encoding BLG (pBLG mice) or with a control plasmid (pcDNA.3 mice). pBLG mice expressed BLG in muscle in its native conformation to a level of 1–2 ng/muscle [27]. This corresponds to 1/50 to 1/100 of the amount of BLG administered in BLG mice. Alum was used as an adjuvant as it favours the presentation of the native structure of the antigen [37]. The immune response was followed over 5 weeks, and the results are presented in figure 1. No anti-BLG antibodies were detected in pcDNA.3 mice. BLG-specific IgE were highly induced in alum-BLG mice, whereas they were not

or very poorly expressed in pBLG mice (fig. 1a). Specific IgG1 were induced in both alum-BLG and pBLG mice, but were significantly lower in the latter group of mice (fig. 1b, $p < 0.05$). The time course and intensity of the specific IgG2a response were similar in those groups, but the response in the pBLG group occurred significantly earlier (fig. 1c). Control BLG mice produced no significant levels of anti-BLG IgE, IgG1 and IgG2a, which suggests a Th1 response in mice immunised i.m. with pBLG, but not with BLG itself.

Fig. 2. Cytokine production after DNA immunisation: spleens from naive mice (□), pBLG-immunised mice (100 µg i.m.; ■), BLG-in-alum-immunised mice (50 µg i.p.; ▨), and BLG-immunised mice (100 ng i.m.; ▩) were removed 5 weeks after immunisation. Cultures of single cell suspensions were performed individually in the presence of BLG (25 µg/ml), PBS or ovalbumin (25 µg/ml) as negative controls, or of Con A (1 µg/ml) as positive control. Supernatants were collected 60 h later, and IFN γ , IL-5 and IL-10 were assayed as described in the Material and Methods section. Mean $\pm$ SEM of 5 mice per group are represented. a, b and c indicate a significant difference ($p < 0.05$) between the pBLG group and the BLG in alum, BLG i.m. and naive groups, respectively. No cytokine production was observed when stimulating splenocytes with PBS or ovalbumin, and ConA stimulation was equivalent for the four groups of mice (data not shown).

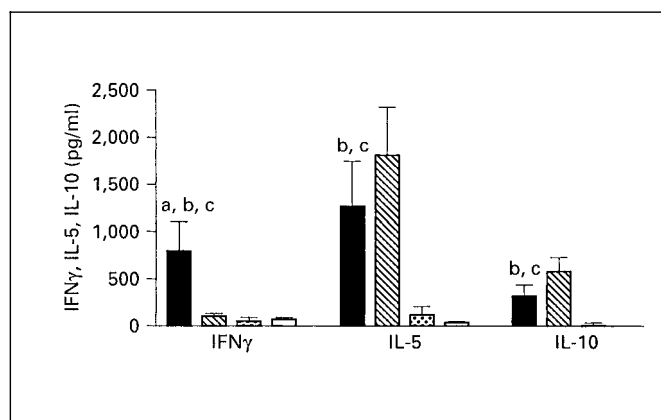
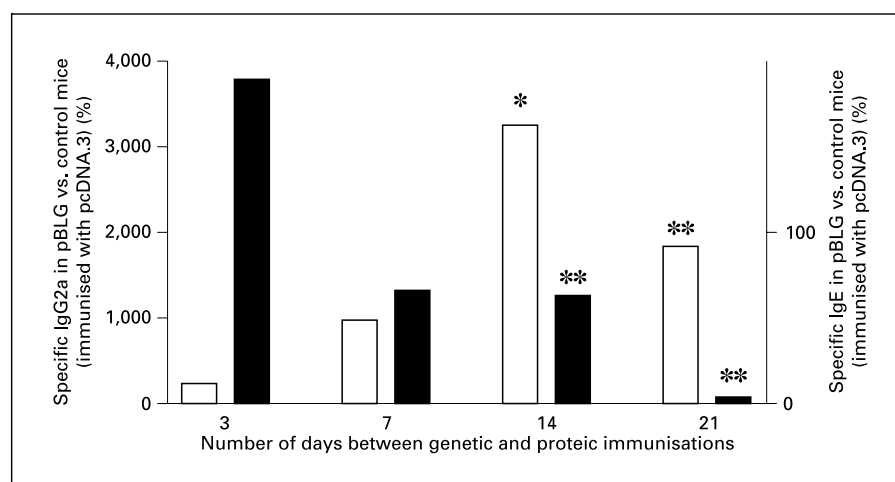


Fig. 3. Determination of the latent period necessary for a preventive effect of DNA immunisation: groups of 4 mice were immunised i.m. with pBLG (or pcDNA.3 as control) and i.p. with 50 µg of BLG adsorbed on alum 3, 7, 14 or 21 days later. Specific IgG2a (□) and IgE (■) were quantified 4 weeks later, and expressed as percentages of specific antibodies induced in pBLG versus control mice (immunised with control plasmid). * $p = 0.07$; ** $p < 0.05$.



Cytokine Production. To verify the Th1 orientation of the immune response in pBLG mice, we analysed the cytokine production after in vitro BLG stimulation of the splenocytes from the different groups of mice. As shown in figure 2, IFN γ was produced in the pBLG group in significantly higher amounts than in the control and alum-BLG groups. IL-4 (data not shown), IL-5 and IL-10 productions were slightly reduced in pBLG versus alum-BLG mice after reactivation, but the differences were not significant ($p > 0.05$).

Preventive Effect of Genetic Immunisation on Th2-Induced Response

Time Dependence of the Preventive Effect of Genetic Immunisation. We first checked for the time necessary to induce a Th1 response in pBLG mice that would persist after a further sensitisation using the protein and possibly overcome the effect of this sensitisation. Mice were thus

previously immunised i.m. with pBLG or with PBS, pcDNA.3, or BLG as controls, and then sensitised using i.p. administration of BLG in alum. These pre-immunisations were performed 3, 7, 14 or 21 days before the sensitisation. Polarisation of the immune response was evaluated 4 weeks after protein sensitisation. The antibody responses are represented in figure 3, as percentages of specific IgE and IgG2a induced in pBLG mice versus control mice. We observed a decrease in specific IgE, correlated with an increase in specific IgG2a, as time between genetic and protein immunisations increased. More than 50% inhibition of IgE production was reached when the latent period was longer than 14 days ($p < 0.05$), whereas the increase in specific IgG2a only became significant after a longer latent period. PBS or BLG control mice had an antibody response similar to that of pcDNA.3 mice (data not shown).

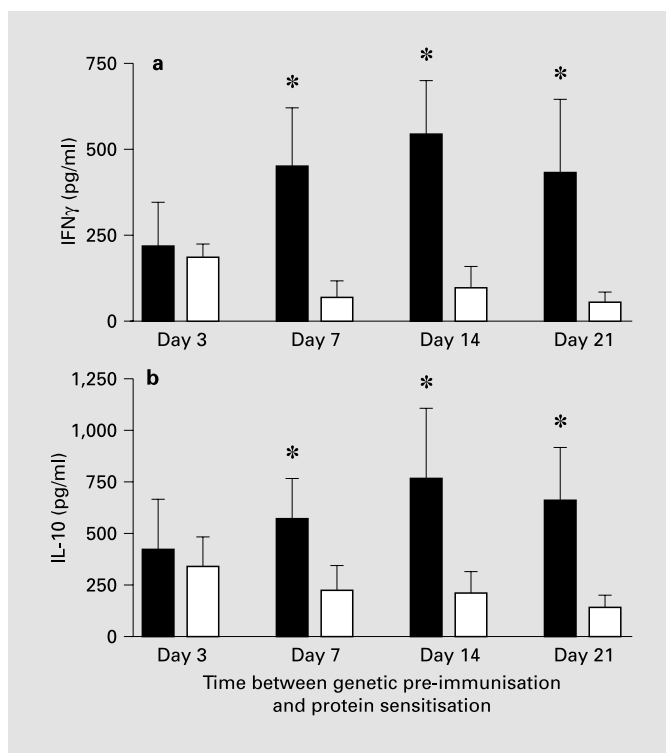


Fig. 4. IFN γ (a) and IL-10 (b) secretions from spleen cells, depending on the latent period between DNA and protein immunisation. Groups of 4 mice were pre-immunised i.m. with pBLG (■) or BLG as a control (□). Three, 7, 14 or 21 days later mice were immunised i.p. with BLG in alum. Seven weeks after sensitisation, spleens were removed and cultured individually in the presence of BLG (25 μ g/ml), ovalbumin (25 μ g/ml) or PBS (negative controls), or of Con A (1 μ g/ml, positive control). No significant cytokine secretion was detectable when stimulating cells with PBS or ovalbumin, and Con A stimulation was equivalent for the two groups of mice. Means $\pm$ SD of 4 mice are given. * = Significant difference between pBLG and BLG-immunised mice in the same latent period group.

Cytokines produced by BLG-reactivated splenocytes were assayed in BLG (control) or pBLG mice then sensitised with BLG in alum. Production of IFN γ (fig. 4a) and IL-10 (fig. 4b) increased greatly in pBLG versus control mice when the latent period increased. IL-5 also decreased in the control group, whereas the production of this cytokine was constant in pBLG-immunised mice (data not shown).

Persistent Effect of the Genetic Immunisation. The time course of the immune response and the persistent effect of genetic immunisation were further analysed. Mice were pre-immunised with pBLG or control plasmid, and 3 weeks later with BLG in alum. A booster injection was given 3 weeks later. No specific IgE (fig. 5a) was pro-

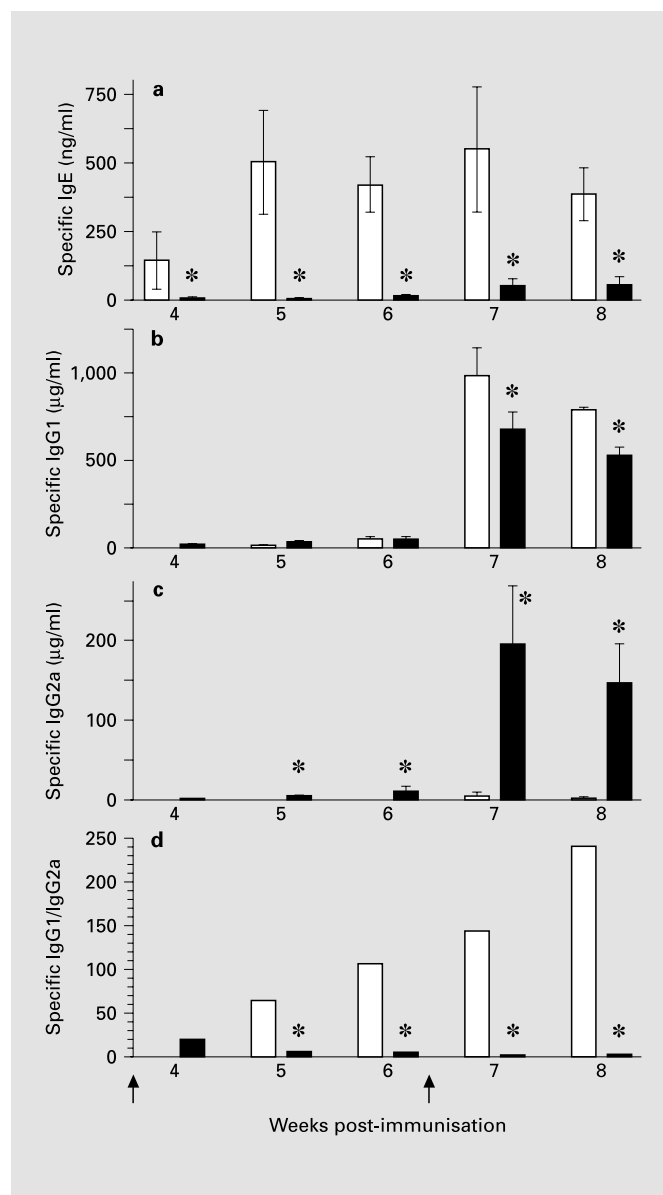


Fig. 5. Persistence of the preventive effect of DNA immunisation. Mice were pre-immunised with pBLG (■) or control plasmid (□), respectively). Three and 6 weeks later, mice received i.p. administration of 50 μ g of BLG adsorbed in alum. Serum-specific IgE (a), IgG1 (b) and IgG2a (c) were quantified weekly, as described in Material and Methods. Specific IgG1/IgG2a ratio is also shown (d). Significant differences are indicated (* $p < 0.05$; t test). Arrows indicate time of sensitisations with the protein.

duced after the first sensitisation, and this inhibition persisted after the booster injection (inhibition $>95\%$, $p < 0.05$). The specific IgG1 response (fig. 5b) was inhibited later, i.e. only after the booster injection, where a 25% decrease was observed in pBLG pre-immunised mice ($p <$

0.05). In contrast, specific IgG2a (fig. 5c) were significantly more induced after the sensitisation and further enhanced after the booster injection in pBLG immunised mice ($p < 0.05$ at weeks 5, 7 and 8 after genetic immunisation). The IgG1/IgG2a ratio was calculated at different times after sensitisation and booster injection (fig. 5d). Assuming that an IgG1/IgG2a ratio above 10 is representative of a Th2 response [38], pre-immunisation with plasmid encoding the allergen allows the induction of a Th1 response that persists after one or two sensitisations with the protein together with the inhibition of the specific IgE response. On the other hand, a booster injection in control mice led to an increase in the specific IgG1/IgG2a ratio, reflecting the induction of a Th2 response.

The observed effect is specific to the allergen encoded by the plasmid: injecting bovine serum albumin adsorbed on alum in pBLG or pcDNA.3 pretreated mice (latent period of 21 days) induced an anti-BSA IgE response which was similar in the two groups of mice (data not shown).

Epitopic Specificity

Depending on the kind of immunisation, we have compared what protein conformations and what linear peptides were recognised by specific antibodies. Antibodies were immobilised on anti-isotype-coated plates, and specific antibodies were revealed using AChE-labelled BLG, rcmBLG (mimicking denatured protein) or BLG-derived peptides (see Material and Methods section). pBLG mice produced IgG1 specific to a native form of the protein, whereas alum-BLG mice showed a larger pattern of recognition. In the latter group, native BLG but also rcmBLG, (11–26), (21–24) and (41–60) peptides were well recognised. IgG2a specificity was roughly the same as that of IgG1. IgE specificity could not be compared as pBLG-immunised mice do not produce specific IgE.

When mice were pre-immunised with pBLG and 3 weeks later with BLG in alum, IgG2a recognition was also mainly specific to a native form of the protein, whereas IgG1 recognition was extended to rcmBLG and (11–26), (21–40) and (41–60) peptides, to a lesser extent than in BLG in alum pre-immunised mice.

Discussion

Our study demonstrates that the primary response in mice immunised with pBLG is of the Th1 type. i.m. injection of pBLG induced a primary response characterised by a very low level of IgE, as previously described after

multiple i.d. administration of β -galactosidase-encoding plasmid [18] or after single administration of plasmid-encoding Der p 5 [21]. However, compared to the studies cited above, specific IgG1 levels appear to be higher in our study, and the time course and magnitude of the specific IgG2a response are also different. In fact, IgG2a levels were high in the first weeks post-immunisation and then decreased, in contrast to previous studies showing that IgG2a levels were maintained [18, 19, 21]. These differences may be due to the fact that BLG is partially secreted, whereas the non-secreted forms of proteins were used in most previous studies evaluating the effect of DNA immunisation on the allergen-specific immune response [18, 21, 22, 24, 25]. In fact, we previously quantified *in situ* BLG production after i.m. administration of pBLG. Those assays demonstrated that BLG is produced in a native form in tibialis muscle. Moreover, *in vitro* and *in vivo* studies demonstrated that recombinant native BLG is partially secreted by transfected cells [27]. As IL-12 induced by ISS must be present at the antigen presentation site for the Th1 orientation of the response [39], the secreted part of produced protein may escape this Th1 environment. Then i.m. pBLG administration leads to both IgG1 and IgG2a responses (but not to IgE response), unlike the strict IgG2a response observed with non-secreted antigen [40].

It is noteworthy that the efficiency of the preventive effect of pDNA immunisation is directly correlated with the amount of specific IgG2a during the primary immune response and may thus be related to a well-established Th1 response. Specific IgG2a concentrations increased until day 21, which corresponds to the best latent period between pDNA pre-immunisation and protein sensitisation. Our results demonstrate for the first time that prevention of the allergic response by pDNA immunisation is highly dependent on the time lapse between administration of pDNA and protein. The efficiency of the preventive effect (using a single i.m. administration and a latent period of 21 days) on specific IgE response was equivalent to that reported in previous publications using multiple i.d. administrations [18]. Moreover, a single i.m. administration of pBLG led to a not previously described increase in specific IgG2a [21], although the IgE inhibition was nearly similar to our results. It is also worth noting that IgE assays were performed after IgG depletion on protein G, thus avoiding the underestimation of IgE concentrations that occurs when IgE is determined directly on sera, particularly during the second and late response.

Moreover, we found a good correlation between IgG2a induction and IFN γ production by splenocytes. In con-

trast, inhibition of IgE production was not correlated with a significant inhibition of IL-4 secretion. Therefore, it seems that inhibition of IgE synthesis results from an active suppression mechanism rather than from a decrease in IL-4 production. IFN γ has been demonstrated to allow in vivo IgE inhibition, with or without modulation of IL-4 production, which supports our results [41–43]. IL-10 is an immunosuppressive cytokine secreted in mice by Th2 and Tr1 cells [9, 44] and has been demonstrated to inhibit Th1 development in different mouse models [45–48]. On the other hand, IL-10, like IL-6 and IL-12, is induced in vitro by naive spleen cells stimulated by ISS [49], and is involved in the prophylactic inhibition of the Th2 response by CpG oligonucleotides. The role of IL-10 in inhibiting the IgE and IgG1 response in mice induced by ovalbumin in alum was also described using a transgenic mouse model expressing human IL-10 [48]. The observed IgE inhibition could be explained by the increased production of both IL-10 and IFN γ occurring when the period between pDNA and protein immunisations increases.

Epitopic specificity of IgG1 antibodies in pBLG mice pre-immunised and then further sensitised with BLG in

alum suggested that the low Th2 response partly consists of a primary response to BLG, which escaped the preventive effect of DNA immunisation. In contrast, specificity of IgG2a was restricted to native BLG, as it was in pBLG mice. The IgG2a response after BLG-in-alum immunisation can then reflect a strict secondary response to BLG.

In conclusion, we have demonstrated a preventive effect of a single i.m. pre-immunisation with a BLG-encoding plasmid on the induction of a Th2 response and a polarisation toward a Th1 response, after a sensitisation protocol with BLG in alum. This preventive effect is strictly dependent on the timing of the administration of pDNA and protein. It is accompanied by increased IFN γ and IL-10 production by splenocytes, whose exact role has yet to be determined.

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