

***In vivo* kinetics of the immunoglobulin E response to allergen: bystander effect of coimmunization and relationship with anaphylaxis**

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Summary

Background Murine models of hypersensitivity to allergens are useful tools for the evaluation of preclinical strategies to down-regulate the IgE response.

Objective To monitor the long-term kinetics of T and B cell responses to allergen as a function of allergen dosage and to investigate the effect of parallel immunization with a second antigen; to correlate B cell response with anaphylaxis.

Methods CBA/J mice were sensitized every other week by subcutaneous injections of phospholipase A₂ (PLA₂) and/or ovalbumin (OVA) adsorbed to alum. Specific antibody isotype responses, T cell proliferation, T cell cytokine production and anaphylaxis were assessed throughout the sensitization phase.

Results Low-dose immunization with PLA₂ (0.1 µg) favoured a long-term, specific T helper (Th)2 response with high IgE and IL-4 production in contrast to high-dose PLA₂ (10 µg) immunization, which biased the immune response towards a Th1 response with high IgG2a and low IL-4 production. Parallel immunization with an unrelated antigen (ovalbumin) had a significant bystander effect on the immunization with PLA₂, which was also dose-dependent. Finally, although anaphylaxis as measured by rectal temperature drop was allergen-specific, it could be induced in the high- and low-dose immunization groups, and was not solely dependent on IgE levels.

Conclusion Though low-dose allergen immunization appears to induce an efficient IgE response, the intensity and quality of this response may be modulated by bystander effects of parallel immunization and does not correlate strictly with anaphylaxis. This observation has relevance to the design of clinical immunotherapy protocols using murine model-based data.

Keywords mouse, IgE, allergy, Th1, Th2, immunotherapy, anaphylaxis, phospholipase A₂, coimmunization, peptide

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Introduction

Animal models of allergy have largely contributed in gaining new insights into the pathogenesis of allergy and in defining potential strategies for therapeutic intervention. Although numerous murine models of asthma are available [1–6], models describing the kinetics of the allergen-specific IgE response, and the clinical relevance of this response, have been less commonly reported. In 1971, Vaz et al. [7] for the first time described the effect of antigen dose on the induction of IgE, repeated low doses generating high IgE titres, whereas the IgE response was markedly attenuated with high doses. Other investigators [8–12] expanded these findings to elucidate antigen-specific IgE regulation in CBA/J mice. Despite an extensive analysis of the antibody response to the allergen and of the mechanisms

underlying this response, current models of systemic murine hypersensitivity do not offer a longitudinal follow-up of the humoral and cellular events leading to the induction of IgE. Furthermore, there is only little evidence of a possible bystander effect of parallel immunization with a second antigen on another defined allergen [13]. This question is highly relevant to the field of allergen immunotherapy, since current preparations of allergen extracts include multiple allergens in various concentrations, which may exert potentially opposite effects on the immune response. Finally, the systemic pathophysiological relevance of the IgE response, i.e. anaphylaxis upon challenge with the allergen, and its expression over time during the course of sensitization are incompletely known. FcεRI has been shown to be essential for the induction of anaphylaxis in the mouse, whereas FcγRIII played important amplifying functions [14–16]. If FcεRI were instrumental in inducing anaphylaxis, IgE were not the only antibodies able to activate mast cells, since IgE knock-out mice developed anaphylaxis of comparable severity as wild-type animals [17]. Part of the role of IgE in inducing anaphylaxis may have been taken

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over by IgG1, though there is yet no definitive answer to this hypothesis.

In this paper, we focused our interest on the *in vivo* kinetics of the specific humoral and cellular responses to phospholipase A₂ (PLA₂) a major bee venom allergen, and examined whether this response might be influenced by parallel immunization with another unrelated allergen, in a dose-dependent manner. We furthermore examined how anaphylaxis was related to the IgE response over time upon challenge with the allergen. Low-dose immunization with PLA₂ favoured a long-term, but late specific Th2 response in contrast to high-dose PLA₂ immunization, which biased the immune response towards a Th1 response. Parallel immunization with an unrelated antigen (ovalbumin (OVA)) had a significant and dose-dependent bystander effect on the antibody response to PLA₂. Finally, anaphylaxis was allergen-specific, could be induced in the high- and low-dose immunization groups, though in a different time-course, and did not appear solely dependent on IgE. A better understanding of the kinetics of low-dose repeated immunizations, which by some aspects mimic allergen exposure, and of high-dose repeated immunizations, which may be compatible with desensitization protocols in allergic patients, may help to more precisely define how to intervene to modify the course of the immune response in the most efficient way.

Materials and methods

Reagents

PLA₂ was purchased from Latoxan, Rosans, France. IgG1 anti-PLA₂ mAb 1A was kindly provided by Dr W.-D. Müller, Klinikum der Friedrich-Schiller Universität Jena, Jena, Germany, and used as control. Overlapping long peptides mapping the entire PLA₂ molecule (LP₁₋₆₀, LP₄₇₋₉₉, LP₉₀₋₁₃₄) [18] were synthesized applying a solid phase F-moc procedure on an Applied Biosystems (Foster City, CA, USA) 431A Peptide Synthesizer, as described [19].

Mice and immunization protocols

Six-eight weeks-old-female CBA/J mice were obtained from Harlan (Zeist, Netherlands). Animals were sensitized by injecting every other week PLA₂ (0.1 or 10 µg) or equimolar doses of OVA (0.28 or 28 µg) (Fluka Chemie AG, Buchs, Switzerland) adsorbed to 1 mg alum (Al(OH)₃) (kindly provided by Dr G. DelGiudice, Chiron-Vaccines, Siena, Italy). Control animals were injected with PBS-alum 1 mg. When coimmunizations were performed, both PLA₂ (high dose 10 µg, low dose 0.1 µg) and OVA (high dose 28 µg, low dose 0.28 µg) were mixed prior to adsorption to 1 mg of alum. A total of one prime and five booster injections were administered subcutaneously (s.c.) at two sites (base of the tail and ventrally). Animals were bled before each immunization by tail vein incision. For the induction of active anaphylactic responses, sensitized animals were injected i.p. with either 30 µg of PLA₂ (maximal non-toxic dose) or 280 µg of OVA. A similar protocol was used for the induction of passive anaphylactic responses, except that in this case naïve CBA/J mice were first injected i.p. with 600 µL of serum from PLA₂ sensitized animals (pool of six mice with strong IgE response), then challenged i.p. with 30 µg of PLA₂. Rectal temperature was subsequently monitored with a calibrated digital thermometer.

Specific antibody determination

Sera were analysed for the presence of PLA₂- or OVA-specific antibodies by ELISA. Briefly, 96-well ELISA plates (Nunc, Maxisorp, Roskilde, Denmark) were coated overnight at 4°C with 5 µg/mL PLA₂ or OVA in carbonate/bicarbonate buffer (0.1 M NaHCO₃, pH 9.6). After three washes with phosphate buffer solution (PBS), plates were blocked in 1% bovine serum albumin (BSA)-PBS for 1 h at 37°C. For specific anti-PLA₂ IgE determination, sera were diluted 1:25 in PBS/Tween (PBS/T)/1% BSA and incubated for 1 h at 37°C prior to incubation with biotinylated rat anti-mouse IgE 3-11 (IgG1, kind gift from Dr C. Heusser, Novartis, Basel, Switzerland) diluted 1:5000. After five washes in PBS/T, plates were incubated with alkaline-phosphatase-conjugated ExtrAvidin (Sigma, Buchs, Switzerland), 1:10000 in PBS/T-1% BSA. Para-nitrophenyl-phosphate (Sigma) dissolved at 1 mg/mL in diethalonamine buffer was utilized as substrate. Optical densities were measured at 405 nm using a microtitre plate reader (Dynatech, Microtec SA, Embrach, Switzerland). An anti-phosphorylcholine IgEκ mAb aPC 12-3 (from Dr C. Heusser) was used as standard antibody for IgE quantification in duplicate wells coated with phosphorylcholine-conjugated BSA (20 µg/mL) and developed as described above.

A similar protocol was used for the determination of specific anti-PLA₂ IgG isotype titres. Sera were serially diluted by two-fold dilutions starting from 1:100 in 1% BSA/PBS/T. Biotinylated rabbit anti-mouse IgG1 and IgG2a were diluted 1:3000 (Caltag, WBAG Resources, Zürich, Switzerland) and finally incubated with alkaline-phosphatase-conjugated ExtrAvidin as above. Titres were expressed as the logarithm of the last dilution exhibiting a threefold higher optical density when compared with preimmune sera.

Cell proliferation assays

Two weeks after the final administration of PLA₂ or PBS, lymph nodes (inguinal, axillary, para-aortic) and spleens were isolated and gently dissociated in cell culture medium containing RPMI 1640 (Seromed, Biokrom KG; Berlin, Germany) supplemented with 2 mM l-glutamine (Gibco, Life Technologies AG, Basel, Switzerland), 10 mM HEPES buffer (Gibco Life Technologies AG), 10% fetal calf serum (FCS; Inotech AG, Dottikon, Switzerland), 100 IU/mL Penicillin/Streptomycin (Gibco Life Technologies AG), 20 µg/mL Gentamicin Sulphate (Sigma) and 5×10^{-5} M β-Mercaptoethanol (Fluka, Buchs, Switzerland). For proliferation assays, cells from various lymph nodes were pooled and cell suspensions (200 µL at 10^6 cells/mL) incubated in round-bottom 96-well plates (Dynatech, Microtec SA, Embrach, Switzerland). For PLA₂ and PLA₂ peptide solutions (used as a mixture of the three long peptides at identical concentration), final concentrations ranged from 0.1 to 10 µg/mL. Concanavalin A (10–2.5 µg/mL Pharmacia Biotech AG, Dübendorf, Switzerland) was used as positive control. Cells were incubated for 72 h in a humidified incubator at 37°C, 5% CO₂ and pulsed with 1 µCi of ³(H)-thymidine (NEN Life Sciences, Brussels, Belgium; 6,70 Ci/mmol specific activity) per well during the last 14 h. Cells were then harvested on filters (Canberra Packard AG, Zürich, Switzerland) and ³(H)-thymidine incorporation into DNA was determined with a scintillation β-counter (Topcount, Canberra Packard AG). Results were expressed as stimulation indexes.

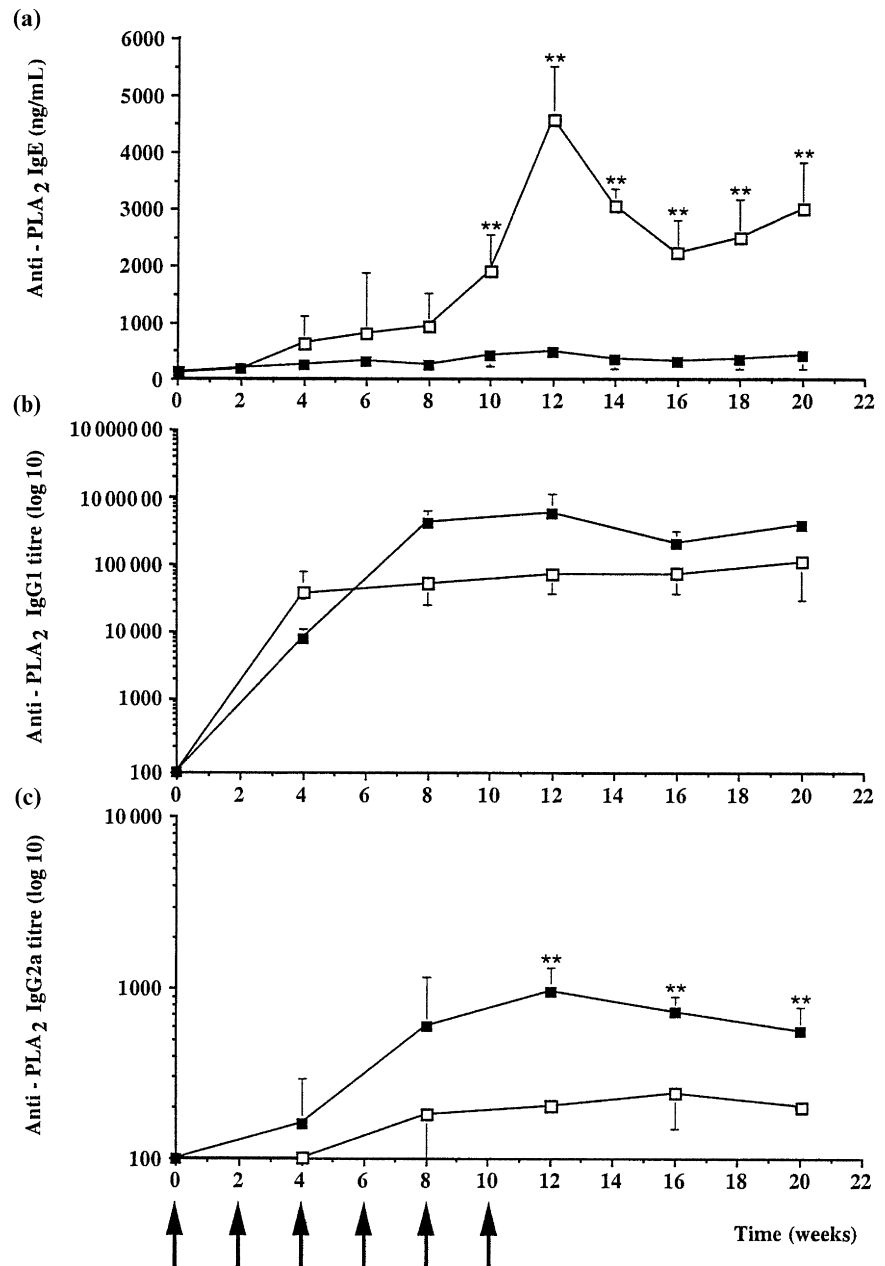


Fig. 1. Serum anti-PLA₂ antibody response is dependent on the allergen dose used for immunization. PLA₂ 10 µg (■) or 0.1 µg (□) was repeatedly injected at weeks 0, 2, 4, 6, 8 and 10 (arrows). Before each immunization, sera were collected and PLA₂-specific IgE (a), IgG1 (b) and IgG2a (c) antibodies determined by ELISA. Groups of five animals were analysed and results from one representative experiment out of four are expressed as mean SD. ***P* < 0.001 by Student's *t*-test (two-tailed) (high-dose (10 µg) vs. low-dose (0.1 µg)).

Cytokine secretion

For cytokine measurements, 1 mL cell suspensions adjusted to 4×10^6 cells/mL were cultured in 24-well plates (Costar, Integra Biosciences AG, Wallisellen, Switzerland) in the presence or the absence of 10 µg/mL PLA₂-derived peptides (used as a mixture at identical concentration) or concanavalin A. Cytokine production in unstimulated and antigen-driven cell cultures was measured after 48 h using a cell incorporating ELISA (ceELISA) as described by Beech et al. [20]. Briefly, 96-well plates (Maxisorp Immunoplates, Nunc, Roskilde, Denmark) were coated overnight at 4°C with the following anti-cytokine capture antibodies at 2 µg/mL and 100 µL per well in carbonate buffer (0.1 M NaHCO₃, pH 9.6): anti-IL-4 (11B11; Pharmingen, Hamburg, Germany) and anti-IFN-γ (01 E70 3B2; kindly

provided by Dr J. Louis, WHO, Epalinges, Switzerland). After washing, plates were blocked with medium containing 10% FCS for 1 h at 37°C. After two washes with PBS, duplicate recombinant cytokine standards (rIL-4, X6310 and rIFN-γ, L1210; from Dr J. Louis) were serially diluted. Cell culture samples were added at 100 µL per well and incubated for 24 h at 37°C in a humidified atmosphere, 5% CO₂. Plates were washed with PBS/T before detection of cytokines by incubating at room temperature (20–25°C) for 2 h with the following biotinylated anticytokine antibodies (50 µL per well): anti-IL-4, 0.5 µg/mL (BVD6–24G2; Pharmingen, Hamburg, Germany), anti-IFN-γ, 1.2 µg/mL (AN1; from Dr J. Louis). Plates were then washed six times with PBS/T before adding the substrate *p*-nitrophenyl-phosphate (Sigma) dissolved at 1 mg/mL in diethanolamine buffer.

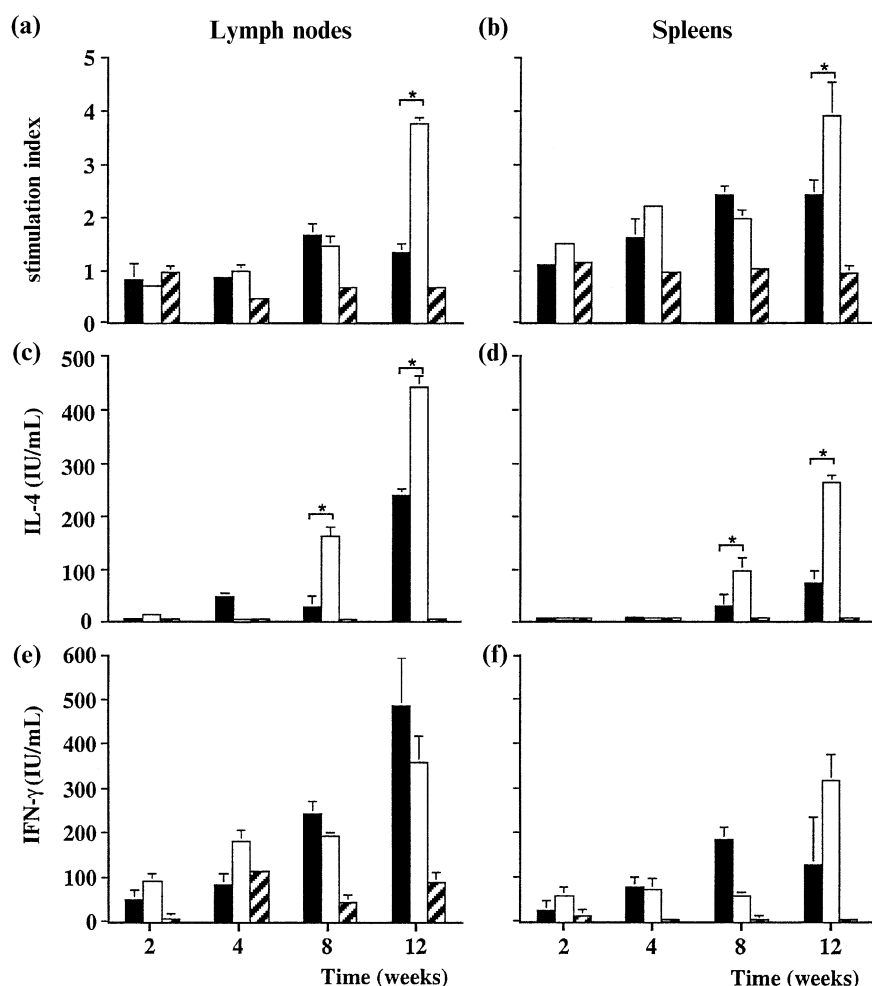


Fig. 2. Dose-dependent antigen-specific T cell proliferation and cytokine production in lymph nodes and spleen during sensitization. Groups of five mice were sensitized with 10 μ g of PLA₂ (■), 0.1 μ g of PLA₂ (□) or PBS (hatched). Lymph node (a, c and e) and spleen T cells (b, d and f) were stimulated with a mixture of the three long synthetic overlapping peptides (10 μ g/mL each) derived from PLA₂ (LP₁₋₆₀, LP₄₇₋₉₉ and LP₉₀₋₁₃₄) at weeks 2, 4, 8 and 12. T cell proliferation in response to PLA₂ peptides was expressed as stimulation indices (a and b). Cytokine production (IL-4: panels c and d; IFN γ panels e and f) in culture supernatants was measured using a cell incorporating ELISA. Data is expressed as mean $\pm$ SD. * P = 0.001 by unpaired Student's t -test (two-tailed).

Statistical analysis

Differences among different groups of mice for antibody induction and anaphylactic responses were examined for statistical differences as indicated by the paired or unpaired Student's t -test (two-tailed) using the InStat software, Mac version 2.01, 1993, with P < 0.05 as the limit of significance.

Results

Allergen-specific polarized B cell response is dose-dependent

To evaluate the dose-dependency of specific IgE, IgG1 and IgG2a antibody response after immunization with high and low PLA₂ doses, CBA/J mice were injected subcutaneously (s.c.) six times every other week with, respectively, 10 or 0.1 μ g of alum-adsorbed PLA₂. Low PLA₂ doses (0.1 μ g) generated a strong specific IgE response that peaked at week 12 and reached a plateau thereafter (Fig. 1a). At least six consecutive injections were necessary to reach maximal and stable IgE levels that persisted for up to 50 weeks (data not shown). In contrast, mice that had received a 100-fold higher dose (10 μ g) of PLA₂ had 10–100 times lower circulating specific IgE levels (Fig. 1a).

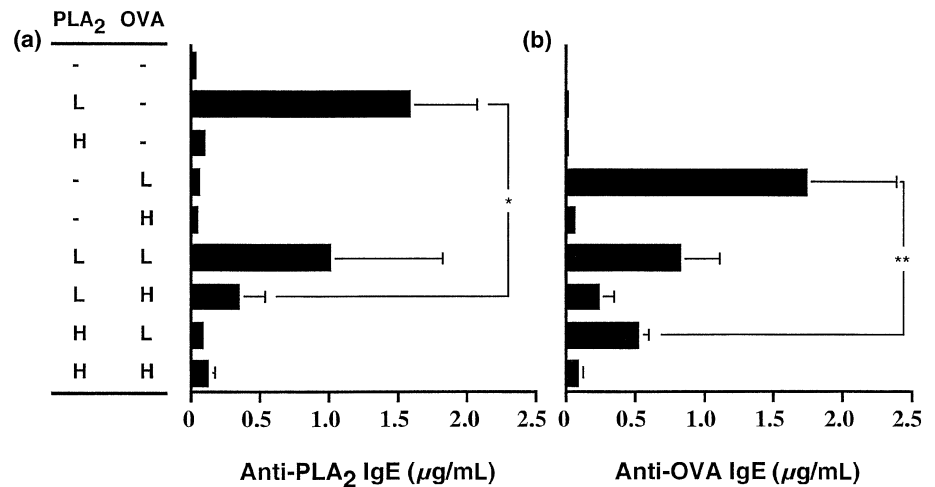
PLA₂-specific IgG1 titres were elevated in both groups (Fig. 1b), and did not differ significantly, whereas IgG2a

response was directly related to the administered PLA₂ dose (Fig. 1c). A competition between specific IgE and IgG for PLA₂ epitope binding was excluded by preadsorbing immune sera on protein G-Sepharose prior to performing IgE-specific ELISA. Though IgG binding to PLA₂ was completely abrogated upon incubation with protein G-Sepharose, no difference in IgE binding was observed between protein G-adsorbed and non-adsorbed sera (data not shown). This experiment thus excluded that the low anti-PLA₂ IgE titres observed after high-dose PLA₂ immunization were merely the result of a binding competition between anti-PLA₂ IgE and high anti-PLA₂ IgG titres.

Antigen-specific T cell proliferation and cytokine production during sensitization with PLA₂ are dose-dependent

Antigen-specific T cell proliferation and cytokine secretion were assessed in lymph nodes and spleens at weeks 2, 4, 8 and 12 during the course of sensitization with either 10 μ g or 0.1 μ g PLA₂. In the low-dose group (0.1 μ g), antigen-specific T cell proliferation to PLA₂ long peptides (10 μ g/mL) steadily increased over the observation period to peak at week 12 (Fig. 2a). In contrast, in the high-dose group (10 μ g), the proliferative response remained low. Differences between the two groups became significant late during the course of sensitization at week 12, with persistently low proliferation indexes in the

Fig. 3. *In vivo* antigen-specific IgE response is modulated by coimmunization with an unrelated antigen. Mice ($n=5$) were immunized with equimolar concentrations of coadministered antigens, as indicated on the legend panel on the left. H (high-dose antigen): PLA₂ 10 µg or OVA 28 µg; L (low-dose antigen): PLA₂ 0.1 µg or OVA 0.28 µg; -: no antigen. Fifteen days after the last immunization (week 12), sera were collected and analysed for PLA₂-specific (panel a) and OVA-specific (panel b) IgE antibodies. Data is expressed as mean $\pm$ SD. * $P=0.04$, ** $P=0.001$ by Student's *t*-test (two-tailed).



high-dose group both in lymph nodes and in the spleen as compared to the low-dose immunization group (Figs 2a and b). Similar results were obtained with detoxified native PLA₂ (data not shown).

The kinetics of IL-4 production in response to PLA₂ differed between the two groups, with a vigorous and sustained secretion from week 8 in animals that had received low-dose sensitization. In contrast, in the high-dose group (Figs 2c and d), peak IL-4 secretion occurred later, at week 12, but was nonetheless non-negligible. The kinetics of IFN- γ production did not markedly differ between the two groups, steadily increasing over the 12 weeks (Figs 2e and f). A typical Th1 or Th2 pattern of cytokine secretion was established only relatively late in the course of immunization, at week 8, both for the high-dose (IL-4/IFN- γ ratio: 0.11) and for the low-dose group (IL-4/IFN- γ ratio: 0.83). Globally, this kinetics, both in terms of T cell proliferation or cytokine secretion, was similar in lymph nodes and spleen T cells.

Parallel immunization with an unrelated antigen exerts a non-specific bystander effect on specific B cell responses to PLA₂

In order to assess the effect that two unrelated antigens may exert on their reciprocal B cell responses, PLA₂ and OVA were coadministered in different combinations of doses. At week 12 of the immunization protocol, repeated administrations of low PLA₂ doses alone induced an expected high specific IgE level (Fig. 3a). This specific response was partially inhibited (36%) by coadministering a low OVA dose, and was significantly suppressed (77%) by coimmunization with a high OVA dose ($P=0.04$). A similar down-regulation of the specific IgE response to OVA could be observed in mice coinjected with low (52%) or high PLA₂ doses (70%) (Fig. 3b). A full time-course of the coimmunization experiments is presented in Fig. 4. Interestingly, as previously observed at week 12 in Fig. 3 for IgE, and all along the follow-up period in Figs 4a and b, similar effects could be noted on the specific IgG1 and IgG2a response: generally, the high equimolar dose of OVA extinguished the PLA₂ response (Figs 4c and e). Conversely, the high PLA₂ dose partially, but significantly down-regulated the OVA response (Figs 4d and f). These observations thus indicate that though the dose of a given allergen may profoundly affect the polarization of the immune response, coimmunization with an unrelated antigen

may further exert a significant non-specific bystander effect on this response.

The kinetics of active systemic anaphylaxis in sensitized animals is related to the sensitizing dose of allergen

Anaphylaxis in mouse is associated with a marked decrease in body temperature [14,15]. This feature was utilized here to assess the physiopathological relevance of the IgE induction upon immunization with low-dose allergen. Upon i.p. challenge with the allergen used for immunization, sensitized mice exhibited a striking drop in rectal temperature, which was maximal 30 min after injection (Fig. 5). Other signs of anaphylaxis developed in parallel: reduced activity, tachypnea, lethargy, and, in some cases, death. In contrast, naïve controls and mice sensitized with control allergen remained at their basal level of rectal temperature when challenged. This clearly indicated that systemic anaphylaxis was allergen-specific. During the course of systemic sensitization, the kinetics and intensity of the anaphylactic reaction was related to the dose of the sensitizing allergen, as illustrated in Fig. 6. Indeed, mice that were sensitized with high doses of PLA₂ dropped their rectal temperature upon i.p. challenge with soluble allergen as early as two weeks after the first immunization with PLA₂ (Fig. 6a). Over the course of immunization, this initial temperature drop had a tendency to decrease in intensity, although this group of mice never reached complete protection from anaphylaxis. In the low-dose group, the onset of the anaphylactic response occurred later, at week 4 only, after at least the priming injection and one booster injection. Passive anaphylaxis could be reproduced by preinjecting week 12 sera from mice immunized with low-dose PLA₂ to naïve mice (Fig. 6b). Anaphylaxis was completely abrogated by serum preadsorption on Protein G (Fig. 6b). This data strongly suggested that the kinetics of the anaphylactic reaction was related also to the dose of the allergen used for sensitization. Furthermore, the anaphylactic reaction was not merely the reflect of circulating IgE levels.

Discussion

In this longitudinal *in vivo* study, we were primarily interested in characterizing the kinetics of the allergen specific antibody response and the parallel secretion of two T cell cytokines

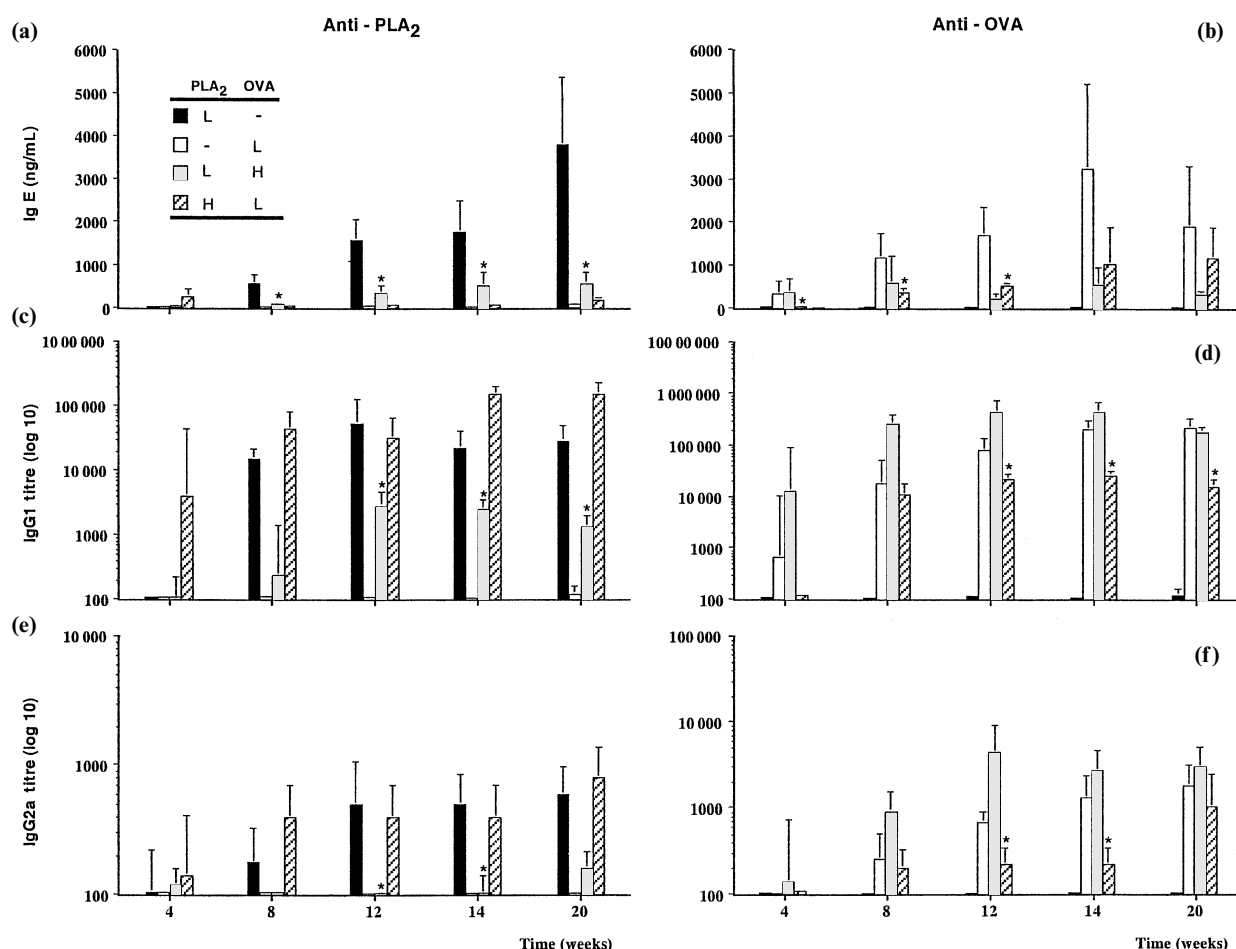


Fig. 4. Kinetics of *in vivo* antigen-specific antibody isotype response in a model of antigen coimmunization. Mice ($n=5$) were immunized with equimolar low (L) or high (H) concentrations of coadministered antigens, as indicated in the legend panel. H (high-dose antigen): PLA₂ 10 µg or OVA 28 µg; L (low-dose antigen): PLA₂ 0.1 µg or OVA 0.28 µg; -: no antigen. Sera were analysed for PLA₂-specific (panels a, c and e) and OVA-specific (panels b, d and f) IgE, IgG1 and IgG2a antibody response over the immunization (week 0–10) and postimmunization period (week 12–20). Data is expressed as mean $\pm$ SD. * $P < 0.05$ by Student's *t*-test (two-tailed) comparing (L-) vs. (L H) for anti-PLA₂ antibodies and (L-) vs. (H L) for anti-OVA antibodies, respectively.

playing a crucial role in IgE regulation (IL-4) and/or representative of the polarization of the immune response towards a Th1- or Th2-type of response (IL-4/IFN- γ ratio) [21]. Previously, several groups had shown that the intensity of the IgE response was dependent on the dose of allergen used for immunization [7–10]. However, the time-course of this response and the kinetics of the secretion of the cytokines that supported it were still unclear. We here confirmed that the *in vivo* antibody response was largely influenced by the dose of the allergen at term (Fig. 1). After low-dose antigen injection via the subcutaneous route, the IgE response was slow to increase, to finally reach levels significantly higher than those induced by high-dose immunization after week 8 only, and to peak at week 12. This IgE kinetics is consistent with the relatively slow increase in IL-4 secretion by PLA₂-specific T cells in the low-dose group both in lymph nodes and in the spleen, as compared to an earlier rise in IFN- γ in both groups.

Several mechanisms have been implicated in the programming of the T cell cytokine response upon stimulation with the allergen. It has been well demonstrated in allergic patients

in vitro that the CD4⁺ T cell production of IL-4 in response to the allergen was dependent on the concentration of the antigen, but also on the type of antigen-presenting cells involved in this response [22,23]. Furthermore, the intensity of the T cell receptor engagement appears to contribute to the cytokine pattern of human allergen-specific T helper cells [24,25]. *In vivo* and *in vitro*, the involvement of costimulatory molecules B7.1 or B7.2 may drastically direct the response towards a Th1- or Th2-type of cytokine secretion [26,27]. We show here *in vivo* that the T cell response leading finally to a Th2-type of response was established only late in the course of low-dose antigen immunization (week 8–12), and was even preceded by an earlier rise in IFN- γ secretion (from week 4) comparable to the high-dose immunization group (Fig. 2). This pattern of cytokine production was reproduced both in T cells isolated from lymph nodes and from the spleen. It did correlate with parallel moves in IgG or IgE production as mentioned above, and thus appears functionally relevant. At peak IgG or IgE response (week 12), high-dose allergen immunization was indeed associated to a low IL-4/IFN- γ ratio, and low-dose allergen

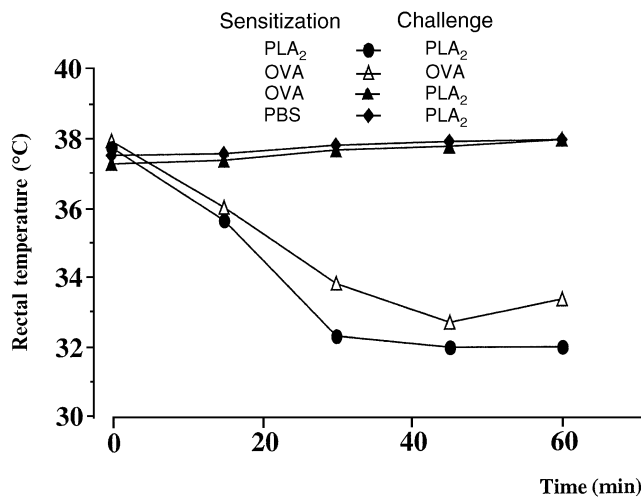


Fig. 5. Systemic anaphylaxis is antigen-specific. Mice were sensitized with PLA₂ 0.1 µg, or with an equimolar concentration of OVA (0.28 µg) or PBS. Two weeks after the last immunization, animals were challenged i.p. with either 30 µg PLA₂ or with OVA (28 µg). Rectal temperature was recorded 15, 30, 45 and 60 min after the challenge. Data is expressed as mean rectal temperature of three mice per group; SD for each group were < 1°C and omitted for clarity.

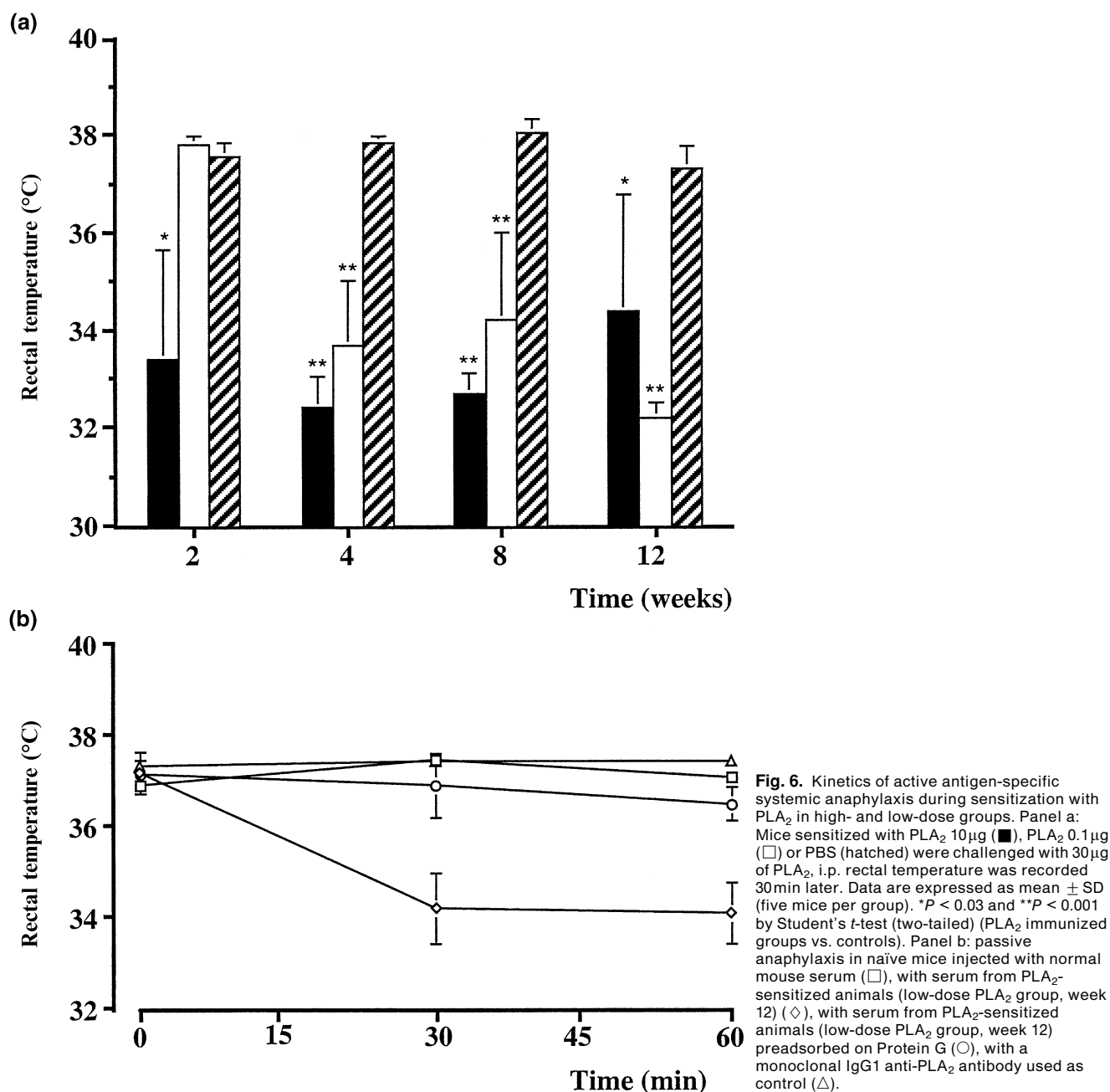
immunization to a relatively higher IL-4/IFN- γ ratio. Clearly, in the low-dose group, T cell priming in the presence of IFN- γ was not sufficient to impair final T cell programming towards a Th2-type of response, an observation consistent with *in vitro* models [28]. The fine control of this cytokine kinetics is yet unexplained. As suggested by *in vitro* studies, one might speculate on the dynamic role of various subsets of antigen-presenting cells [22], and the parallel production of other cytokines crucial for the orientation of the response such as IL-12 [29–31], or the involvement of specific sets of costimulatory molecules [26]. Thus, in contrast to previous findings [32], we here show that the high IgE response was supported by a high IL-4/IFN- γ ratio, and the low IgE response by a low IL-4/IFN- γ ratio; however, this trend was only established late in the course of immunization.

In vitro, allergen-specific T cells from allergic patients proliferated poorly to low-dose antigen, despite marked parallel production in IL-4 [22]. *In vivo*, we here observed with T cells isolated from both lymph nodes and spleen that repeated s.c. administration of high-dose antigen led to a plateau in cell proliferation after 8 weeks already, though with low-dose immunization the stimulation indexes steadily increased. This situation is reminiscent of a state of anergy induction such as obtained with so-called supraoptimal concentrations of antigen [33], a T cell non-responsiveness or hyperresponsiveness which is not incompatible with Th1-type cytokine secretion [34].

Allergen immunotherapy is usually considered as a specific modulation of the immune response to antigen, and consists in repeated administration of high doses of allergen [35]. We here show that such an approach in a murine model may be significantly altered by the administration of a second antigen in parallel, and by the dose of this latter antigen (Figs 4 and 5). This bystander effect was reciprocal, and was observed both after immunization with PLA₂ or OVA. This suggests that a specific response to an antigen may be modulated non-specifically by the bystander activation of T cell subsets specific for another antigen, or eventually by the non-specific activation

of antigen-presenting cells, which in turn may result in specific or non-specific polarization of cytokine secretion. Globally, the final quality of the specific response in terms of antibody isotype production correlated to the total amount of antigen administered, i.e. low-dose immunization inducing a Th2 response, and high-dose dominating over low-dose by inducing a Th1 response. This observation appeared true not only for IgE, but also for IgG1 and IgG2a production (Fig. 4). In general, high-dose OVA strongly dominated over PLA₂, whereas PLA₂ had a slightly weaker effect on OVA. Since the number of T cell epitopes present on OVA are largely superior to those displayed by PLA₂, whose molecular weight is 3-fold smaller, this may suggest an effect of dominance of OVA by the recruitment of a large population of T cells, eventually competing for a limited amount of cytokines. These findings may have important consequences for therapeutic immunomodulatory approaches such as allergen desensitization. On the one hand, allergen immunotherapy may potentially alter the outcome of parallel immunizations such as vaccination with bacterial antigens, or may itself be modulated by bystander immunization. If parallel immunization involves adjuvants promoting a strong Th1-type of cytokine response, it may even contribute to the final goal of allergen immunotherapy. This bystander effect has generally not been fully recognized in humans, though it was recently shown that during conventional immunotherapy, responses to tetanus toxoid may be affected by allergen desensitization [36]. In an heterologous immunization system using the glutathione S-transferase of the parasite *Schistosoma mansoni* as immunogen in the mouse, coimmunization with Der p 1 had the ability to modify the profile of the immune response by modulating the balance between the polarizing cytokines IL-4 and IFN- γ [13]. A bystander effect of so-called dominant epitopes or non-dominant epitopes was also observed in a model of nasal immunotherapy [37] and further supports our observations.

We finally examined the pathophysiological relevance of the IgE response by evaluating the anaphylactic response to a challenge with the native allergen both in low- or high-dose immunization groups. We here used the decrease in body temperature (rectal temperature) as a surrogate clinical marker of anaphylaxis, as previously suggested [14,15]. We first demonstrated that though the quality of the B cell response may be affected by parallel immunization with a second antigen, the anaphylactic response was clearly allergen-specific (Fig. 5). However, the kinetics of the anaphylactic reaction over the course of immunization was quite remarkable. Already very early in the course of immunization (week 2) with high-dose antigen, a challenge with native allergen induced a profound drop in rectal temperature as compared to control or low-dose immunization groups, which did not significantly differ. Interestingly, the decrease in body temperature became progressively less marked with further high-dose PLA₂ administration, whereas the low-dose PLA₂ immunization was associated with reactions of increasing severity (Fig. 6). This shows that anaphylaxis may be induced in a completely opposite fashion in two groups of primed animals independently of their level of specific IgE, since at week 2, in both groups, IgE levels were comparable. If the mechanisms of anaphylaxis in the low-dose group are related to the increasing generation of specific IgE, the mechanisms of early anaphylaxis in the high-dose group are still undefined. They may rely on soluble factor(s), since transfer of serum to naïve mice could



induce passive systemic anaphylaxis after challenge (Fig. 6). It has been well demonstrated in mice that anaphylaxis is not solely dependent on IgE, and may occur in IgE knockout animals [17]. The possibility to activate mast cells, basophils or other Fc receptor-bearing cells, and to induce anaphylaxis has been clearly demonstrated in FcεRI knockout models expressing FcγRIII [14,15]. A role for IgG1 has been suggested though not demonstrated [17]. The abrogation of the capacity of transferred serum to allow passive anaphylaxis by preincubation with protein G strongly suggests that indeed immunoglobulins may be involved in the induction of body temperature drop. It has however, to be noted that the level of IgE, IgG2a or IgG1 were comparable in the low- and high-dose groups at week 2. This may indicate further involvement of other yet

undetermined soluble factors including complement or even cytokine themselves. One may postulate that IFN-γ, initially secreted at low level, later at high level in the high-dose group, may have a protective effect on the induction of anaphylaxis. This may be supported by the observation in the field of allergen-specific immunotherapy that mastocytes or basophils are less excitable upon high IFN-γ level exposure [38,39].

In conclusion, we here demonstrate that though low-dose allergen immunization appears to induce a vigorous IgE response at term, and high-dose allergen immunization a comparatively low IgE response, the establishment of a stable Th1 or Th2 cytokine pattern occurred late in the course of immunization. Furthermore, though the T cell response to allergen was specific, this response could be significantly polarized (Th1 vs.

Th2) by parallel immunization with a second unrelated antigen. Finally, anaphylaxis did not correlate with IgE level solely, and may involve so far undefined soluble factors, among which non-IgE allergen specific antibodies or cytokines. These observations will have to be taken into account in the analysis of murine models of immunotherapy and may also help to define novel strategies for clinical immunotherapy protocols.

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