

Inducing Tolerance by Intranasal Administration of Long Peptides in Naive and Primed CBA/J Mice¹

Mireille Astori,*[†] Christophe von Garnier,* Alexander Kettner,[†] Nathalie Dufour,*
Giampietro Corradin,[†] and François Spertini^{2*}

To assess the capacity of a peptide-based immunotherapy to induce systemic tolerance via the nasal route, we designed three long overlapping peptides of 44–60 aa covering the entire sequence of phospholipase A₂ (PLA₂), a major bee venom allergen. Both prophylactic and therapeutic intranasal administrations of long peptides to PLA₂-hypersensitive CBA/J mice induced specific T cell tolerance to the native allergen. In prophylactic conditions, this tolerance was marked by a suppression of subsequent specific IgE response, whereas the therapeutic approach in presensitized mice induced a more than 60% decrease in PLA₂-specific IgE. This decline was associated with a shift in the cytokine response toward a Th1 profile, as demonstrated by decreased PLA₂-specific IgG1 and enhanced IgG2a levels, and by a decline in the specific IL-4/IFN- γ ratios. T cell transfer from long peptide-tolerized mice to naive animals abrogated the expected anti-PLA₂ IgE and IgG1 Ab response, as well as specific T cell proliferation, but enhanced specific IgG2a response upon sensitization with PLA₂. These events were strongly suggestive of a clonal anergy affecting more profoundly Th2 than the Th1 subsets. In conclusion, these results demonstrate that allergen-derived long peptides delivered via the nasal mucosa may offer an alternative to immunotherapy with native allergens without the inherent risk of systemic anaphylactic reactions. Moreover, long peptides, in contrast to immunotherapy strategies based on short peptides, have the advantage of covering all potential T cell epitopes, and may represent novel and safe tools for the therapy of allergic diseases. *The Journal of Immunology*, 2000, 165: 3497–3505.

Systemic allergen immunotherapy with whole bee venom (BV)³ provides protection within 3–5 yr in over 80% of patients (1–3). Although the mechanisms of desensitization are still incompletely known, they appear to be associated with a Th2 to Th1 cytokine shift, with a decrease in the levels of allergen-specific IgE, and with a marked decrease in T cell response to the allergen, eventually leading to T cell tolerance (4–8). This may, directly or indirectly, contribute to decrease mast cell or eosinophil activation and to improve patient protection upon reexposure to the allergen (9). Because of possible severe anaphylactic reactions, allergen immunotherapy has to be conducted under close clinical supervision (10). There is thus a strong interest in developing novel strategies, which might be at least as efficient, but safer, of shorter duration, and noninvasive. In this respect, oral or nasal administration of native allergens has been tested in various murine models, and has led to the induction of a successful T cell tolerance (for review, see Ref. 11). In humans, although evidences for clinical efficacy of mucosal immunotherapy are still limited, recent reports suggested that sublingual administration of native allergens

was also able to modulate inflammatory markers in allergic rhinitis and to induce T cell tolerance to the allergen (12, 13).

Several recent studies have evaluated the efficacy of systemic desensitization based on short dominant T cell epitopes derived from allergens in humans (8, 14–16). The major advantage of short peptides is their inability to cross-link IgE on mast cells, therefore avoiding the risk of systemic adverse reactions inherent to conventional immunotherapy with native allergens. However, the T cell response to a given allergen and the major T cell epitopes may vary considerably among patients (8, 16–18). An immunotherapy strategy based on short dominant epitopes may thus require a customized approach, and the tedious characterization of major HLA-restricted T cell epitopes on a patient's basis (17). To overcome this difficulty, long peptides (LPs) of 44–60 aa were designed, covering the whole sequence of phospholipase A₂ (PLA₂), a major BV allergen. We have previously shown that these LPs were able to induce a vigorous T cell response in BV-hypersensitive patients (6). Since LPs contain all possible T cell epitopes, an immunotherapy strategy based on LPs would help to overcome the need for an individual definition of patient's dominant T cell epitopes. Importantly, these long overlapping peptides did not bind IgE, or only in a minority of patients for peptide 90–134 (18), and did not induce skin hyperreactivity in intradermal tests (Fellrath et al., manuscript in preparation). These preliminary data thus suggested that PLA₂-derived LPs could be used safely as substitute for the native allergen.

In this study, based on a murine model of hypersensitivity to PLA₂, we demonstrate that PLA₂-derived LPs, administered via the nasal route, can efficiently induce systemic tolerance after both prophylactic and therapeutic approaches. Furthermore, as a preliminary step to clinical investigation, we show that LPs overlapping the entire sequence of an allergen cover all the dominant T cell epitopes, and may safely be administered without risk of anaphylaxis or intrinsic cytotoxicity.

*Division of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; and [†]Institute of Biochemistry, University of Lausanne, Epalinges, Switzerland

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² Address correspondence and reprint requests to Dr. François Spertini, Division of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois, BH18, Rue du Bugnon, 1011 Lausanne, Switzerland. E-mail address: Francois.Spertini@chuv.hospvd.ch

³ Abbreviations used in this paper: BV, bee venom; i.n., intranasal; LP, long peptide; PLA₂, phospholipase A₂.

Materials and Methods

Animals

Female CBA/J mice (H-2^k) 5–6 wk old were obtained from Harlan Olac (Zeist, Netherlands) and used at the age of 7–8 wk. Animals were maintained under standard housing conditions.

Peptide synthesis and purification

Three long synthetic peptides, LP_{1–60} (LP1), LP_{47–99} (LP2), and LP_{90–134} (LP3), mapping the entire 134 aa of PLA₂ from *Apis mellifera*, were synthesized on an Applied Biosystems 431A Peptide Synthesizer (Perkin-Elmer, Norwalk, CT) (Fig. 1A) (19). A histidine tail at the N-terminal site of the peptides allowed their purification on a nickel column (Qiagen, Chatsworth, CA) and then on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden), as previously described (19). Peptides were fully reduced by two successive 12-h incubations at 37°C, first in 10% 2-ME, second in 6 M guanidium chloride with a 200 molar excess of DTT. They were finally purified on a Sephadex G-50 column. Analytical HPLC and mass spectrometry were used to assess the purity of each peptide (>80%), which was readily soluble in PBS.

Systemic immunization protocol

Mice were sensitized a total of six times every other week by s.c. injections of 0.1 μ g of PLA₂ (Latoxan, Rosans, France) in alum. One day before each injection, serum samples were collected, and the kinetics of the IgE, IgG1, and IgG2a Ab response was determined by ELISA.

Intranasal treatment

Groups of five mice were lightly anesthetized using i.p. injection of pentobarbital (1.2 mg/mouse). As indicated for each experiment, PLA₂ (10, 1, or 0.1 μ g) or a mixture of the three LPs (300, 100, or 10 μ g/peptide) in 30 μ l PBS was administered intranasally (i.n.) for 3 consecutive days. To this purpose, 15 μ l of the Ag solution was gently applied to each nostril and readily aspirated by the animal. Control mice received PBS only. In the therapeutic experiments, PLA₂-sensitized mice were treated i.n. 14 days after the last immunization with PLA₂, whereas in the prophylactic experiments, naive mice were treated i.n. first, and 14 days later sensitized with PLA₂.

PLA₂ purification and detoxification for cell culture

PLA₂ (Latoxan) was purified by HPLC. Its cytotoxicity on cell cultures was inhibited by overnight reduction at 37°C with a 100 molar excess of DTT, followed by alkylation with a 1000 molar excess of *N*-ethylmaleimide. PLA₂ was finally purified on a Sephadex G-25 column (Pharmacia).

Culture medium

Splenic and lymph node cells were cultured in RPMI 1640 (Seromed, Biokrom KG, Berlin, Germany) supplemented with 50 μ M 2-ME (Fluka, Buchs, Switzerland), 2 mM L-glutamine, 10 mM HEPES, 100 IU/ml penicillin-streptomycin (Life Technologies, Basel, Switzerland), 20 μ g/ml gentamicin sulfate (Sigma, Buchs, Switzerland), and 10% FCS (Inotech AG, Dottikon, Switzerland).

Lymphocyte proliferation assays

Mice were sacrificed, and spleens or lymph nodes (inguinal, paraaortic, and axillary) were removed and placed in ice-cold PBS. After gentle dissociation, cellular suspensions were cultured in a volume of 200 μ l in 96-well round-bottom plates at the density of 1×10^6 cells/ml. Reduced and alkylated PLA₂ (10 μ g/ml), or a mixture of the three LPs (10 μ g/ml of each peptide) were added into the culture medium. All proliferation assays were pulsed after 54 h of culture with 1 μ Ci [³H]thymidine/well (Du Pont, NEN Products, Boston, MA), and cells were harvested 18 h later. [³H]Thymidine incorporation in duplicated samples was measured using a Microplate Scintillation Counter (Canberra Packard, Zürich, Switzerland). Background counts for medium alone were 500 cpm $\pm$ 15%.

Cytokine assays

In parallel to lymphocyte proliferation assays, single-cell suspensions of lymph nodes or spleens were cultured as above in a volume of 1 ml in 24-well flat-bottom plates at the concentration of 2×10^6 cells/ml with PLA₂ (10 μ g/ml) or a mixture of the three LPs (10 μ g/ml of each peptide). IL-4 and IFN- γ productions were measured after 48 h by cell ELISA, as previously described (20). Briefly, 96-well Maxisorp plates (Maxisorp Immunoplates; Nunc, Roskilde, Denmark) were coated overnight at 4°C with

2 μ g/ml of anti-IL-4 mAb (11B11; PharMingen, San Diego, CA) or anti-IFN- γ mAb (01E703B2; kindly provided by Dr. J. Louis, WHO, Epalinges, Switzerland) in carbonate/bicarbonate buffer, pH 9.6. After washing, plates were blocked with 10% FCS in culture medium for 2 h. The 48-h stimulated lymph node or splenic cells were gently resuspended, transferred to the plates in duplicates, and incubated overnight at 37°C in 5% CO₂. After extensive washes with PBS/0.05% Tween, plates were incubated first for 2 h at 37°C with biotinylated anti-IL-4 mAb (BVD6-24G2; 2 μ g/ml; PharMingen) or anti-IFN- γ mAb (AN1; 1 μ g/ml; from Dr. J. Louis), then for 30 min with alkaline phosphatase-conjugated ExtrAvidin (1:10,000; Sigma). The enzymatic reaction was developed in the presence of *p*-nitrophenylphosphate and read at 405 nm. Supernatants from clones X6310 (IL-4) and L1210 (IFN- γ) (both from Dr. J. Louis), which were calibrated respectively against recombinant murine IL-4 (PharMingen) or IFN- γ (Life Technologies, Buchs, Switzerland), were used as standards.

Cell transfer experiments

Splenic T cells were purified using magnetic activated cell sorter (MACS) system, as recommended by the supplier (Dynal, Oslo, Norway). Sorted cells were 97% T cells, respectively, 63% CD4⁺, and 34% CD8⁺, as determined by FACS analysis using FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 mAbs (PharMingen). Cells were washed in PBS, resuspended, and injected i.v. to recipient naive CBA/J mice.

Ab isotype determination in sera

Anti-PLA₂ IgG1 and IgG2a responses were titrated by ELISA. Plates were coated overnight at 4°C with 50 μ l of PLA₂ (5 μ g/ml in carbonate/bicarbonate buffer, pH 9.6) and blocked in 1% BSA/PBS for 2 h at 37°C. Sera were serially diluted by 2-fold dilutions in 1% BSA/0.05% Tween in PBS and incubated for 2 h at 37°C. After washing, biotinylated rabbit anti-mouse IgG1 or IgG2a Abs (Caltag, San Francisco, CA) were added for 30 min at 37°C. Assays were revealed by the addition of alkaline phosphatase-conjugated ExtrAvidin (1:10,000) for 30 min at 37°C, and finally of *p*-nitrophenylphosphate (Sigma). OD was determined at 405 nm on a microtiter plate analyzer (MR5000; Dynatech Laboratories, Chantilly, VA). Titers were expressed as the reciprocal of the last dilution superior to 2-fold the preimmune serum OD.

Detection of Ag-specific IgE

ELISA plates (Maxisorp; Nunc) were coated overnight at 4°C with 50 μ l of PLA₂ (5 μ g/ml in carbonate/bicarbonate buffer, pH 9.6) and blocked in 1% BSA/PBS. Sera were diluted 1/25 in 1% BSA/0.05% Tween in PBS. After an overnight incubation at 4°C, plates were washed with PBS/0.05% Tween and incubated for 2 h at 37°C with 50 μ l of biotinylated rat anti-mouse IgE mAb (3-11; kindly provided by Dr. C. Heusser, Novartis, Basel, Switzerland). The assay was then developed, as described above, for specific IgG isotype determination. A biotinylated anti-phosphorylcholine murine IgE mAb (aPC12-3; from Dr. C. Heusser) was used as standard on microtiter plates coated with phosphorylcholine-BSA (20 μ g/ml).

Immunoblotting

Equimolar dilutions of PLA₂ and of each of the three LPs were separated by 15% SDS-PAGE in nondenaturing and denaturing conditions, and transferred onto polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA) in CAPS/methanol buffer (10 mM CAPS, 10% methanol, pH 11). Membranes were blocked for 2 h at room temperature in 1% BSA/PBS, pH 7.4, and incubated overnight at 4°C with the immune sera serially diluted in 1% BSA/PBS/0.05% Tween. Preimmune sera were used as negative controls. After washing in PBS/0.05% Tween, the following biotinylated anti-mouse Abs were used: anti-IgE (3-11) mAb diluted 1/5000 and goat anti-IgG (Caltag) diluted 1/3000. After 1-h incubation at room temperature, membranes were washed and incubated for 30 min at room temperature with streptavidin-HRP conjugate (1/3000; Upstate Biotechnology, Lake Placid, NY). The assay was revealed by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, Little Chalfont, U.K.).

Statistical analysis

A standard Student *t* test was conducted using Instat software Mac version 2.01.

Results

Mapping of the murine T cell response to PLA₂-derived LPs

Three LPs spanning the entire 134 aa of PLA₂ (Fig. 1A) were synthesized as described in *Materials and Methods*. First, to determine their *in vitro* capacity to stimulate PLA₂-specific T cells, CBA/J mice were sensitized by s.c. injections of 0.1 μ g of native PLA₂ in alum six times every other week. This protocol was previously shown to induce high levels of specific IgE and IgG1 Abs in this susceptible strain of mice (21). Lymph node or splenic T cells from PLA₂-sensitized mice proliferated equally well both to PLA₂ and to a mixture of the three LPs in equimolar concentration (Fig. 1B). As shown in separate analyses of the T cell response to each of the LPs, the major T cell epitopes were located mainly within the first 47 N-terminal aa of PLA₂, on the LP1 peptide (Fig. 1B). LP2 and LP3 contributed only in a minor proportion to the proliferative response. In contrast, when the Ab response is analyzed, PLA₂-specific IgE and IgG localized B cell epitopes only on LP3, the PLA₂ C-terminal peptide spanning aa 99–134 (Fig. 1C).

Prophylactic *i.n.* administration of native PLA₂ induces a specific Th1 cytokine secretion

To assess the capacity of the nasal route to modulate the specific T cell response in naive CBA/J mice, PLA₂ was first administered *i.n.* for 3 days before systemic sensitization. As indicated in Fig. 2A, repetitive s.c. immunizations (0.1 μ g of PLA₂ in alum) of PLA₂-pretreated mice (10 μ g group) induced only a negligible rise in PLA₂-specific IgE over a 12-wk time-course experiment, a rise that became later even undetectable ($p < 0.05$ compared with control PBS-treated mice). This inhibition of IgE secretion was dose dependent, since mice pretreated with 1 or 0.1 μ g of PLA₂ did not significantly differ from control mice treated with PBS. Nasal delivery of more than 10 μ g of PLA₂ was toxic, and in most cases even lethal for the mice. While the specific anti-PLA₂ IgG1 response was inhibited by 60–80%, the specific IgG2a response increased by 20–50% during the whole 34-wk time-course analysis (Fig. 2A). These results strongly suggested a long lasting modulation of the T cell response toward a Th1 cytokine secretion profile. Again, the strongest inhibition (up to 30%) of T cell proliferation in response to PLA₂ at wk 12 was obtained in mice pretreated *i.n.* with the highest tolerable dose of PLA₂ (10 μ g) (Fig. 2B). Interestingly, animals that had received lower doses of PLA₂ showed higher T cell stimulation indexes when compared with PBS-treated mice. At the same time point, wk 12, the analysis

of IL-4 and IFN- γ secretion and the determination of their ratio confirmed a shift from a Th2 to a Th1 phenotype (Fig. 2C). This shift was also the most significant ($p < 0.05$) in animals pretreated with the highest dose of PLA₂.

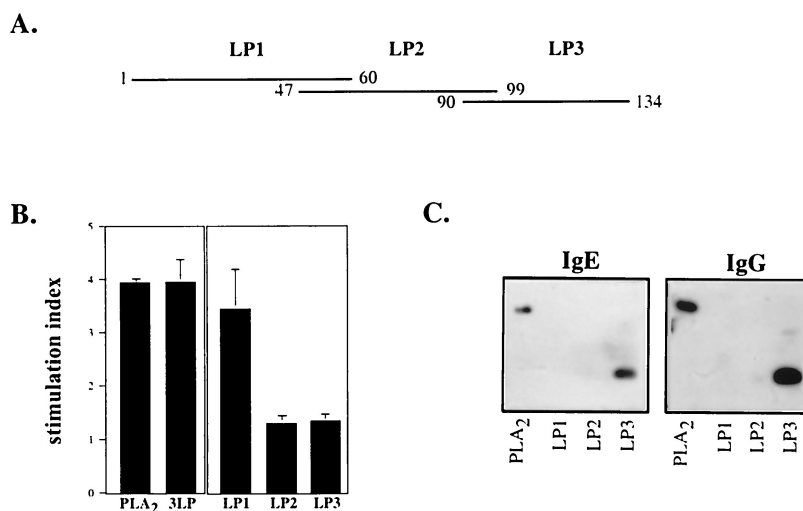
Prophylactic *i.n.* administration of LPs induces T cell tolerance

We then examined whether the three PLA₂-derived LPs could also induce an efficient T cell refractoriness (22), as shown above for native PLA₂. Intranasal administration of the three LPs before systemic sensitization with PLA₂ abrogated the expected specific IgE response as efficiently as native PLA₂ itself (Fig. 3A). During the whole time-course analysis, the strongest inhibition of the Th2-dependent IgG1 secretion was obtained with the highest dose of the peptide mixture (100 μ g of each peptide). This inhibition was superior to 81% when compared with PBS and accompanied by a parallel up to 58% increase of the Th1-dependent anti-PLA₂ IgG2a response. At wk 12, prophylactic nasal treatment with 100 μ g of the three LPs completely inhibited the PLA₂-specific T cell proliferation and IL-4 secretion (Fig. 3, B and C). Some IFN- γ activity could still be detected, although strongly reduced. Here too, there was a clear orientation of the cytokine secretion toward a Th1 pattern, as indicated by a low IL-4/IFN- γ ratio (Fig. 3C). This effect was dose dependent and most prominent with the highest dose of the three LPs (100 μ g).

Therapeutic *i.n.* administration of PLA₂-derived LPs inhibits IgE secretion and T cell activation in PLA₂-sensitized mice

We also evaluated the possibility to modulate the course of an established allergic response by the *i.n.* administration of the three LPs in PLA₂-sensitized mice. Two weeks after the completion of systemic PLA₂ sensitization, as described in *Materials and Methods*, mice were treated *i.n.* for 3 consecutive days with the three LPs, native PLA₂, or PBS. Two weeks later, mice were bled for PLA₂-specific IgE determination. In comparison with specific IgE level before *i.n.* treatment, PLA₂-specific IgE in animals from groups treated with either 300 μ g of each of the LPs or 10 μ g of native PLA₂ decreased by at least 60% ($p < 0.05$) (Fig. 4A). In contrast to the LP therapy, the inhibition of the specific IgE secretion after native PLA₂ treatment was not only accompanied by a strong production of allergen-specific IgG2a, but also of IgG1. T cell proliferation in response to both PLA₂ and the three LPs was markedly diminished (up to 23% and 60%, respectively) (Fig. 4B). This inhibition was dose dependent, as was the shift from a Th2 to

FIGURE 1. Three LPs from PLA₂ efficiently stimulate T cells from primed CBA/J mice. Schematic representation of the three long overlapping peptides, LP1, LP2, and LP3, spanning the entire 134 aa of PLA₂ (A). Lymph node cells from PLA₂-sensitized CBA/J mice ($n = 5$) were stimulated for 3 days with native detoxified PLA₂ (10 μ g/ml), with a mixture of the three LPs (10 μ g/ml of each peptide), or with each of the LPs, LP1, LP2, and LP3, separately (10 μ g/ml). Data are expressed as stimulation indexes (B). Similar stimulation indexes were obtained using splenocytes (data not shown). Lymph node cells or splenocytes from naive CBA/J mice were used as controls. In parallel to the proliferation assay, sera from sensitized mice were analyzed by immunoblots for specific IgE and IgG against PLA₂ and each of the LP (C). Data are representative of three different experiments.



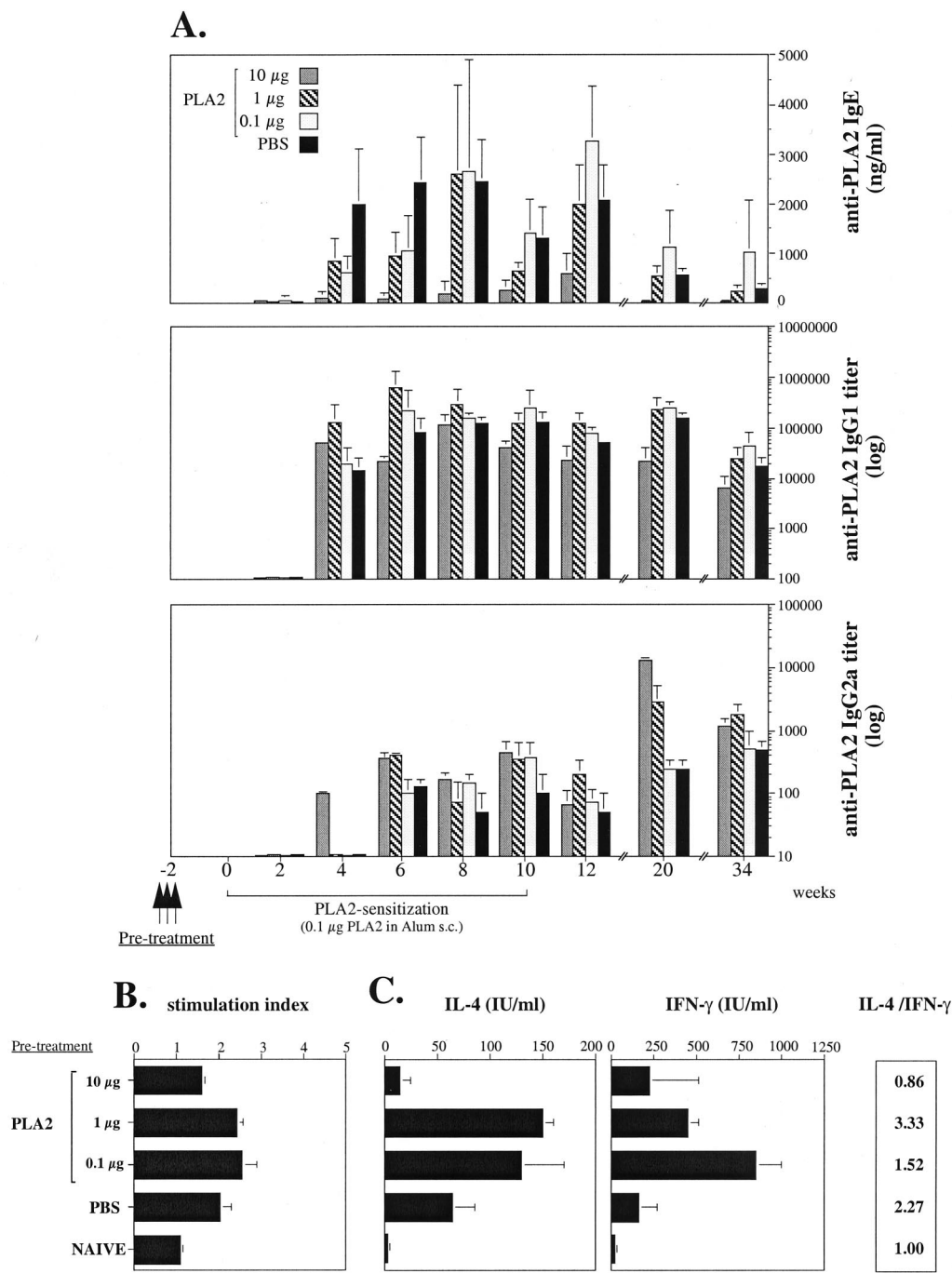


FIGURE 2. Intranasal prophylactic treatment with native PLA₂ inhibits specific IgE response and induces T cell tolerance. Naive CBA/J mice were treated i.n. for 3 consecutive days either with PLA₂ at three different doses (10, 1, or 0.1 μ g) in PBS or with PBS alone as control. Fourteen days after the last i.n. application, animals were sensitized by six consecutive s.c. injections of 0.1 μ g of PLA₂ in alum every other week. Twenty-four hours before each injection, sera were collected for the determination of PLA₂-specific IgE, IgG1, and IgG2a Abs by ELISA (A). Fourteen days after the last PLA₂ injection, 12 wk after the beginning of sensitization, lymph node cells from PLA₂- or PBS-pretreated mice were stimulated in vitro for 3 days with detoxified PLA₂. Data are expressed as stimulation indexes (B). In parallel, IL-4 and IFN- γ production was measured by cell ELISA, and the ratios of IL-4/IFN- γ were determined (C). Parallel stimulation indexes were obtained with splenocytes (data not shown). Naive indicates age-matched CBA/J mice that received no i.n. treatment nor any systemic PLA₂ sensitization and that were kept in similar housing conditions. Data represent the mean $\pm$ SD of five mice per group. The experiment was repeated three times with similar results.

a Th1 cytokine production (Fig. 4C). As compared with the cytokine secretion profile obtained in the prophylactic approach with the three LPs, the production of IFN- γ was here notably enhanced. From Fig. 1, it appeared that PLA₂ major T cell epitopes were mostly located on peptide LP1. We thus attempted to induce T cell tolerance with this peptide alone (300 μ g) in the same therapeutic

setting. This resulted in an average 40% decrease of specific IgE secretion ($p < 0.02$) (Fig. 4A), as well as in up to 52% inhibition of T cell activation (Fig. 4B). Here also the analysis of the IgG2a, IL-4, and IFN- γ secretion established the capacity of this LP to induce by itself a Th2 to Th1 shift (Fig. 4C). These data, however, underline also that T cell epitopes (so-called minor epitopes)

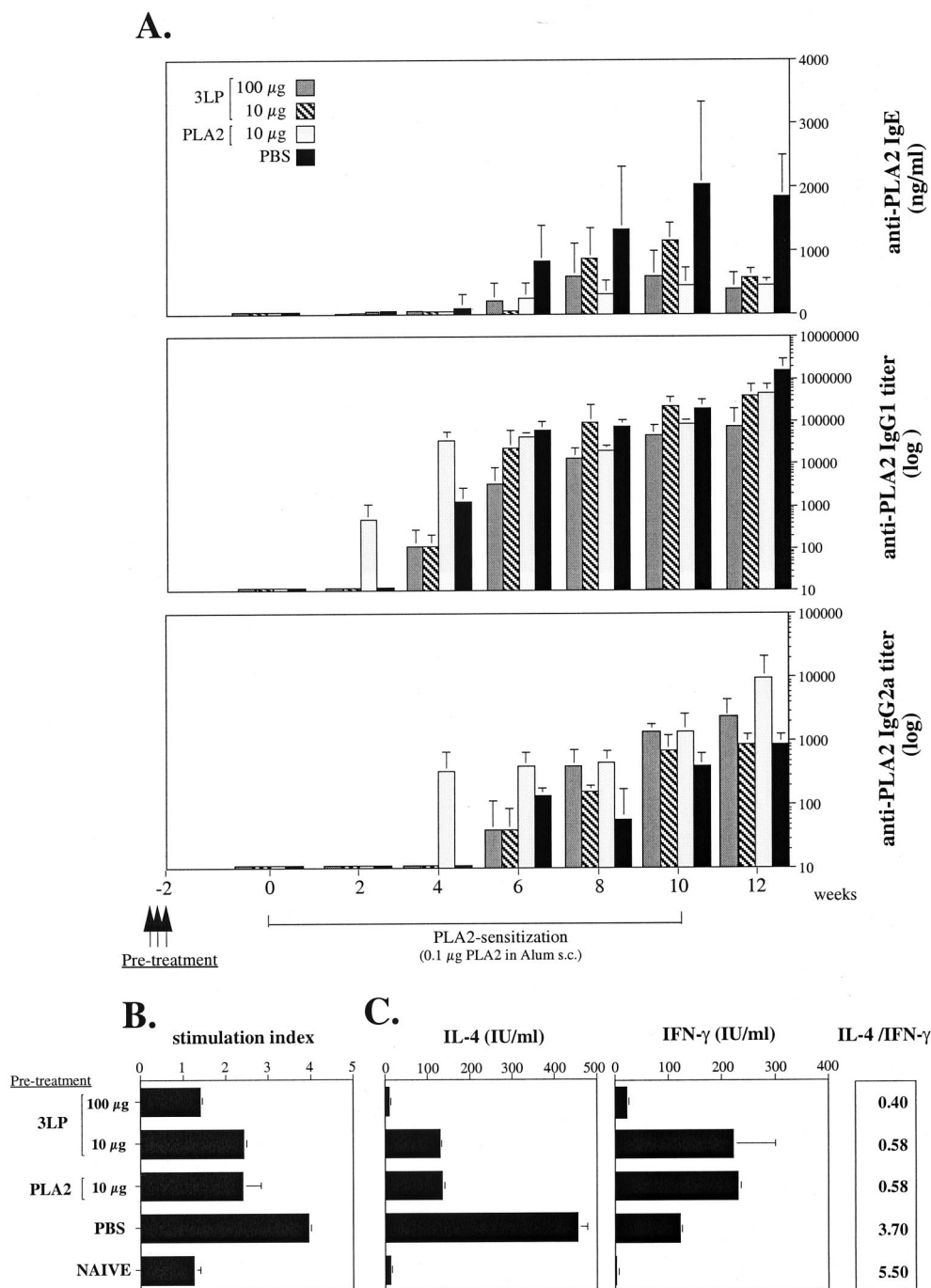


FIGURE 3. Prophylactic i.n. application of LPs efficiently suppresses T cell activation and IgE secretion, upon mice sensitization. Naive CBA/J mice were treated i.n. for 3 consecutive days either with a mixture of the LPs at high (100 μ g of each) or low (10 μ g of each) concentration, or 10 μ g of PLA₂ in PBS. Control animals received only PBS. Fourteen days later, animals were sensitized with s.c. injections of PLA₂, as described in Fig. 2. One day before each PLA₂ injection, sera were collected for anti-PLA₂ IgE, IgG1, and IgG2a analysis by ELISA (A). Fourteen days after the last PLA₂ injection at wk 12, lymph node cells from LP, PLA₂, or PBS-pretreated mice were cultured for 3 days in the presence of detoxified PLA₂. Data are expressed as stimulation indexes (B). In parallel, IL-4 and IFN- γ productions were measured by cell ELISA, and their ratios were determined (C). Parallel analysis was done on splenocytes with similar results (data not shown). In vitro stimulation with the three LPs gave stimulation indexes identical to those of PLA₂ (data not shown). Each result represents the mean $\pm$ SD of five mice per group. Experiments were repeated three times with similar results.

located on LP2 and LP3 did play a role in the more pronounced inhibition of IgE secretion obtained with the mixture of the three peptides.

The delivery of the three LPs on the nasal mucosa was perfectly well tolerated in sensitized mice. In contrast, after each application of native PLA₂ (10 μ g), sensitized mice developed clinical evidence of anaphylaxis, suggested by a sharp decline in rectal temperature and long lasting obtundation (data not shown).

Adoptive transfer of T cells from tolerant to naive mice before sensitization abrogates specific IgE production

The i.n. administration of the three LPs in prophylactic and therapeutic conditions proved to be very effective in inducing tolerance. To examine the role of T cells in this phenomenon, PLA₂-sensitized mice were treated i.n. with the LP mixture, PLA₂, or PBS, as described in the therapeutic experiments formerly reported

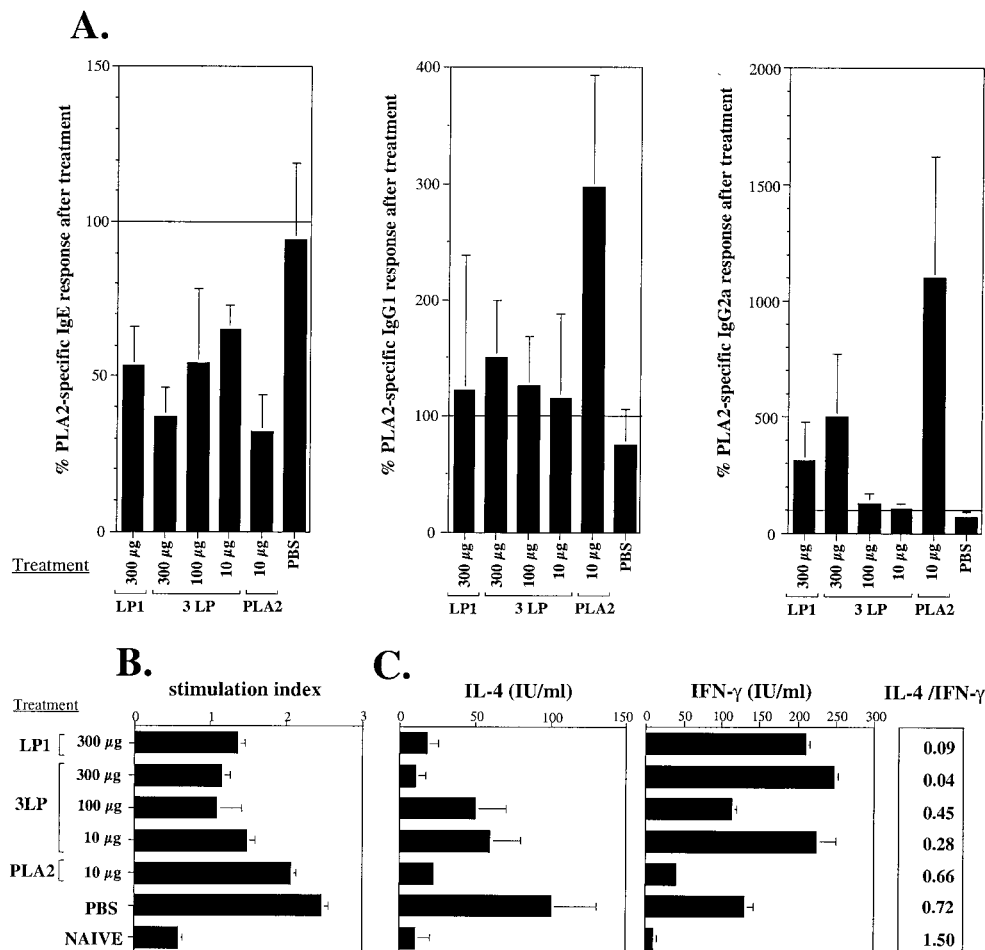


FIGURE 4. Therapeutic i.n. administration of LPs to PLA₂-sensitized CBA/J mice inhibits IgE secretion and T cell proliferation. PLA₂-sensitized CBA/J mice were treated i.n. for 3 consecutive days either with 300 μg of peptide LP1 alone, a mixture of the LPs at three different doses (300, 100, and 10 μg of each) or 10 μg of PLA₂ in PBS. Control animals received PBS alone. Fourteen days after the last i.n. treatment, anti-PLA₂ IgE, IgG1, and IgG2a serum levels were measured by ELISA and expressed as percentage of increase or decrease compared with pretreatment values (A). At the same time point, lymph node cells were cultured for 3 days in the presence of detoxified PLA₂. Results of proliferation assay are expressed as stimulation indexes (B). In parallel, IL-4 and IFN-γ productions were quantified by cell ELISA (C). Data represent the mean ± SD of five mice per group. Experiments were repeated three times with similar results.

in Fig. 4. Splenic CD4⁺ and CD8⁺ T cells were then transferred into naive recipient CBA/J mice. Animals subsequently received four s.c. immunizations with alum-adsorbed PLA₂, 0.1 μg. PLA₂-specific IgE and IgG production, T cell proliferation, and cytokine secretion were measured 2 wk later. The transfer of T cells from animals tolerized with the three LPs completely inhibited the secretion of PLA₂-specific IgE, while supporting an efficient IgG2a production by recipient murine B cells (Fig. 5A). As expected, T cell proliferation (Fig. 5B) and IL-4 secretion (Fig. 5C) were markedly inhibited in parallel. In contrast, transfer of T cells from animals treated with PLA₂ led to even higher IgE and IgG1 responses than T cells from PBS-treated control group.

Discussion

In this study, we have shown that both prophylactic and therapeutic i.n. administrations of PLA₂-derived LPs were able to prevent or respectively inhibit an ongoing PLA₂-specific IgE response as well as the allergen-specific activation of T cells to induce T cell tolerance. The parallel analysis of the specific Ab secretion and cytokine production indicated that pretreatment with allergen-derived LPs induced an early orientation of the T cell response toward a Th1 response. Similarly, we demonstrated for the first time

that treatment of sensitized mice with LPs in contrast to native PLA₂ was able to deviate an ongoing Th2 activation toward a Th1 response. Based on adoptive transfer experiments, we furthermore demonstrated that tolerance could be efficiently transferred to naive CBA/J mice by splenic T cells from tolerized animals. These results support the use of LPs as efficient and safe tools to induce tolerance in allergic diseases. Moreover, the mucosal route, which proved in this study to be effective, may strongly facilitate treatment application.

It is now well established that peripheral tolerance can result from clonal deletion or anergy (23). T cell transfer from peptide-tolerized mice, as described in Fig. 5, was conferring to the recipient mice a resistance to IgE induction despite repeated immunizations with the native allergen. Although we cannot exclude that clonal deletion may have occurred during i.n. treatment with the LPs, these data would rather favor a mechanism of clonal anergy of allergen-specific T cells. Indeed, clonal deletion would have resulted at term in the transferred naive mice in the induction of an allergen-specific immune response. After tolerance induction, we still observed a persistent secretion of IFN-γ, which was accompanied by an increase in specific IgG2a secretion. Such IFN-γ production by anergic CD4⁺ T cells has been previously reported

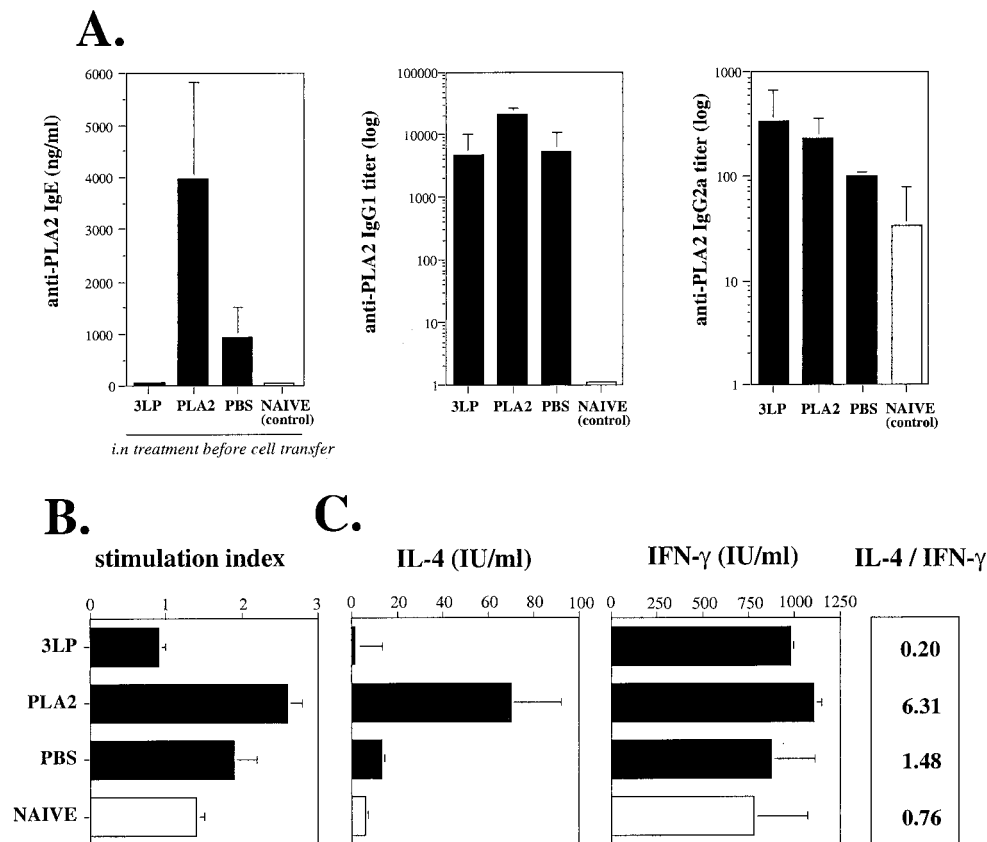


FIGURE 5. T cell transfer from LP-tolerized mice to naive CBA/J mice abrogates anti-PLA₂ IgE induction and confers T cell tolerance. PLA₂-sensitized CBA/J mice (five mice per group) were treated i.n. for 3 consecutive days with either 300 μ g of the LPs, 10 μ g of PLA₂, or PBS. Fourteen days after the last i.n. treatment, CD4⁺ and CD8⁺ T cells were purified from spleens, and 7×10^5 cells were transferred i.v. to naive CBA/J mice. As control, T cells from naive CBA/J mice were transferred to naive CBA/J mice. Two days after cell transfer, the recipient mice were immunized four times every other week by s.c. injections of 0.1 μ g of PLA₂ in alum. Two weeks after the last immunization, serum level of PLA₂-specific IgE, IgG1, and IgG2a was determined by ELISA (A). At the same time point, lymph node cells were cultured for 3 days in the presence of detoxified PLA₂. Results of proliferation assays are expressed as stimulation indexes (B). In parallel, IL-4 and IFN- γ productions were quantified by cell ELISA (C). Data represent the mean $\pm$ SD of three recipient mice per group.

in a model of clonal anergy in a TCR-transgenic mouse (24). Taken together, our data indicate that transferred T cells effectively interfered with the delivery of cognate help by the critical donor T cells to the recipient mice B cells to turn off specific IgE, but not IgG secretion. In systemic models of tolerance, the nature of the processes that lead to the induction of T cell functional unresponsiveness *in vivo* is not yet clear, but the IL-2 production defect associated to T cell anergy appears to result from at least two simultaneous but distinct mechanisms: cytokine-mediated immunosuppression and a block in the activation of early response kinases leading to clonal anergy (25). Recent experimental data indeed have suggested a possible role for regulatory cytokines such as IL-10 in the induction of peripheral T cell unresponsiveness (7, 25–28). Spleen cells from DO11.10 OVA-TCR transgenic mice can be rendered unresponsive when cultured for long period in the presence of Ag and IL-10 (29, 30). Cell lines derived from human and murine CD4⁺ populations cultured in similar conditions were subsequently found to be suppressive for proliferation of naive T cells by secreting IL-10 and TGF- β , and have been called Tr1 cells (30). In addition to IL-10, TGF- β has been implicated as a regulatory cytokine produced by T cells found at sites of chronic antigenic stimulation, and is thought to contribute to the induction of oral tolerance (31, 32).

Soluble protein Ags encountered through the respiratory or gastrointestinal tracts do not elicit strong systemic immune responses,

but induce a state of Ag-specific unresponsiveness that is commonly referred to as mucosal tolerance (33). The down-regulation of immune responses in the gut mucosa is relatively well defined and appears to rely on at least three distinct mechanisms: clonal deletion (34), anergy (35, 36), and active suppression mediated by regulatory cells secreting TGF- β and Th2-like cytokines (23, 33, 37). In contrast, the immunological processes underlying the natural immunity to inhaled proteins are poorly defined. Active tolerance capacity of the respiratory tract mucosa is supported by several independent reports that IgE Ab induction to an immunogenic challenge is inhibited by prior exposure to aerosolized OVA, whereas specific IgG responses remained intact (38, 39). In this respect, it has to be noted that pretreatment and therapeutic approach with native PLA₂ did not prevent a strong IgG1 response in contrast to LPs (Figs. 2, 3, and 4). The enhanced IgG1 response in PLA₂-treated mice may be related to a persistent secretion of IL-4 *in situ* by PLA₂-specific T cells, as a result of a different Ag processing and presentation pathway, potentially by different APCs (40). In addition, the capacity of PLA₂ to cross-link IgE may further enhance IL-4 secretion by mast cells or, as described in rodents, PLA₂ by its intrinsic enzymatic activity may induce an IgE-independent mediator release from mast cells, leading to *de novo* IL-4 synthesis (41, 42). The production of allergen-specific IgG1 observed in this study is consistent with the recent observation that immunotherapy with purified allergens induces mouse IgG1 Abs

that recognize similar epitopes as human IgE, thereby inhibiting IgE/allergen interactions and allergen-induced basophil degranulation (43). Furthermore, transfer of CD4⁺, but not CD8⁺ T cells from i.n. tolerized mice clearly suppressed ongoing Ag-specific IgE, but not IgG1, responses in primed recipients, pointing out differences in the regulation of T cell-dependent Ag-specific IgE and IgG1 responses (39).

Experimental evidence in rodents led to the hypothesis that immunologic homeostasis to inhaled proteins was mediated by a population of Ag-specific $\gamma\delta^+$ CD8⁺ T cells secreting IFN- γ (44). The role of CD8⁺ and $\gamma\delta^+$ T cells is, however, still largely debated. Indeed, the administration of nebulized OVA to CD8⁻ and $\gamma\delta$ -deficient mice reduced IgE responses and blood eosinophilia to subsequent challenges to the same degree as in normal wild-type mice (38). Furthermore, a more recent study demonstrates that the in vivo deletion of CD8⁺ T cells did not prevent the induction of i.n. tolerance (45). In this model of i.n. tolerance in naive mice, the dose-dependent inhibition of T cell expansion was associated with the reduction of both Th1- and Th2-type cytokine secretion (45). Moreover, secretion by spleen or lymph node T cells of immunosuppressive cytokines such as TGF- β and IL-10 was not detected in OVA-unresponsive animals, in contrast to gastrointestinal or systemic tolerance models. The production of IL-10 mRNA by splenocytes or lymph node T cells was analyzed in our model by PCR: we have also been unable to show any enhancement of its production (data not shown). In contrast, in the study by Tsitoura et al. (45), costimulatory pathways between APC and Ag-specific CD4⁺ T cells appeared to play a central role, since in vivo, the inhibition of the interaction of T cells with CD86, but not CD80, at the time of exposure to i.n. Ag, prevented the development of immunological tolerance. Although similar mechanisms may be involved in our model, no data are to date available on the mechanisms of tolerance in an established model of IgE hypersensitivity.

Early studies on mucosal tolerance were restricted to analyses of cellular responses to whole protein Ags, but it now appears that immunogenic peptides that contain T cell epitopes can also act as potent tolerogens in vivo (46–48). Intranasal or oral administration of a single immunodominant peptide derived from the house dust mite protein Der p 1, when given before immunization with the whole protein, can induce peripheral tolerance (49, 50). Systemic or mucosal administration of short dominant epitopes derived from Der p 1 (30, 31), Bet v 1 (32), or Fel d 1 (33) led to the induction of T cell tolerance to the whole allergen in several murine experimental models, in prophylactic settings only, however. The clinical efficacy of such an approach has been recently investigated in humans (16, 34). Allergen-derived peptides or adapted approaches (LPs, mutated allergens, allergen fragments, etc.) may be of the utmost interest for the therapy of allergic diseases, since peptides with low or absent IgE-binding capacity, but conserved recognition by T cells, can be easily synthesized, and offer a safe alternative to native allergen for specific immunotherapy (14, 18, 51–53).

In our peptide-based mucosal immunotherapy model, immune modulation appeared long lasting, as shown by both prophylactic experiments and T cell transfer. Interestingly, the nasal administration of the LP mixture displayed a better capacity to confer T cell anergy after adoptive transfer than the administration of native PLA₂. This may be related to a better presentation of particular tolerizing sequences from peptides or by the involvement of different APC subsets. However, the concentration of PLA₂ and peptides was in this study not comparable (10–30-fold lower for PLA₂), a difference that may have played a crucial role and may not allow a direct comparison between the two approaches. This

underlines another advantage of peptides: the possibility to use them without fear of intrinsic toxicity or anaphylactic reactions at concentrations unacceptable for the native allergen from which they were derived. The therapeutic administration of all three peptides on the nasal mucosa induced a more pronounced inhibition of T cell proliferation than did peptide LP1 alone, a peptide that spans the dominant murine T cell epitopes (Fig. 1). This may be due to the presence of other significant, although nondominant T cell epitopes on the two C-terminal peptides or alternatively to cryptic epitopes on PLA₂ or on the three LPs themselves, which may be presented only after Ag processing (37). The efficacy of LPs in inducing tolerance seems to be related to the total amount of peptides delivered, both in prophylactic and therapeutic protocols. Indeed, the nasal administration of small doses of the LPs was less efficient in inducing T cell tolerance. This observation differs from a previous report on the tolerizing capacity of a short synthetic peptide containing the major T cell epitope of Der p 1 (30). At doses ranging from 1 to 100 μ g, there were no differences in the capacity of the dominant peptide epitope to induce tolerance via the nasal route. Although we do not have a definitive explanation, this discrepancy may be related to the nature of the allergen and to the particular epitopes involved.

In parallel to the induction of T cell tolerance, specific T cells upon LP treatment were still able to secrete IFN- γ and to deviate T cell cytokine profile toward a Th1-type profile. It has been shown in a murine model of asthma, using an adoptive transfer system, that Th1 cells did not attenuate Th2 cell-induced airway hyperreactivity and inflammation in either SCID mice or OVA-immunized immunocompetent BALB/c mice, but rather caused severe airway inflammation (54). However, such models are in part artificial, since in contrast to tolerance induction, they do not involve potential counterregulatory antiinflammatory mechanisms, as observed in systemic tolerance (via the production of IL-10 and TGF- β). Such mechanisms may exist in nasal tolerance, although yet undescribed, perhaps in situ, and may reduce the potential danger of a pure Th1 response. Indeed, in an asthma model, CD4⁺ T cells engineered to express latent TGF- β abolished airway hyperreactivity and airway inflammation induced by OVA-specific Th2 effector cells in SCID and BALB/c mice, in contrast to OVA-specific Th1 cells (55).

In conclusion, this study shows for the first time that long overlapping peptides derived from allergens may substitute for the native protein to induce tolerance via the nasal route, and more importantly were able to modify the course of an established IgE response. T cell tolerance can be transferred to naive mice, which suggests that this phenomenon was T cell dependent and related to T cell anergy and not T cell deletion. In contrast to the native allergen, LPs do not have cytotoxic activity for the respiratory tract even at high doses and do not induce anaphylaxis. Allergen-derived long overlapping peptides may thus be considered as potential candidates for a novel, safe, and effective immunotherapy of allergic diseases.

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