Antigen-Independent Suppression of the Allergic Immune Response to Bee Venom Phospholipase A2 by DNA Vaccination in CBA/J Mice

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Antigen-Independent Suppression of the Allergic Immune Response to Bee Venom Phospholipase A\textsubscript{2} by DNA Vaccination in CBA/J Mice\textsuperscript{1}

Samantha Jilek, Catherine Barbye, François Spertini, and Blaise Cortésy\textsuperscript{2}

Phospholipase A\textsubscript{2} (PLA\textsubscript{2}) is one of the major honey bee venom allergens for humans. To assess the long-term prevention of allergic reactions by DNA vaccination, a PLA\textsubscript{2}-CBA/J mouse model was employed using empty or PLA\textsubscript{2} sequence-carrying DNA plasmids. Early skin application of either DNA construct before (prophylactic approach) or after (therapeutic approach) sensitization with reactions by DNA vaccination, a PLA\textsubscript{2}-CBA/J mouse model was employed using empty or PLA\textsubscript{2} sequence-carrying DNA plasmids.

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\textsuperscript{3} Abbreviations used in this paper: SIT, specific Ag immunotherapy; FLAG, the octapeptide DYYDDDDK; ISS, immunostimulatory sequence; ODN, oligodeoxynucleotide; PLA\textsubscript{2}, bee venom phospholipase A\textsubscript{2}; PBS-T, PBS-Tween 20.

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Ag-specific Th2 cell-mediated responses occurs through the dominant effect of the Th1 background. Together, our data demonstrate that it is possible to successfully select for memory Ag-specific Th cells exhibiting a prevalent Th1 phenotype using independent administration of DNA and Ag.

Materials and Methods

Construction of expression vectors

The expression vector used was pSecTagA (Invitrogen, Groningen, The Netherlands). Among other features, the vector contains a CMV promoter, an Ig γ-chain sequence for protein secretion, and a polyadenylation site. Expression vectors pSecTagA-PLA2 (PLA2_V), pSecTagA-P1 (P1V), pSecTagA-P2 (P2V), and pSecTagA-P3 (P3V) were constructed from pSecTagA (EV) and are coding for the whole bee venom PLA2 and three derived peptides (aa 1–60, aa 47–99, aa 90–134). PCR amplification of the coding regions was conducted using oligonucleotides 1–8 listed in Table I and a PL2_CDNA clone as a matrix (a gift from Mireille Astori, University of Lausanne, Lausanne, Switzerland). The FLAG octapeptide used for detection of the secreted proteins and peptides was inserted at the amino terminus using a second PCR amplification and oligonucleotides 5–8 and PLA2.FLAG (Table I).

Vector pSecTagA-LacZ coding for Escherichia coli β-galactosidase was generated to confirm the efficacy of the expression vector after intradermal DNA vaccination. The β-galactosidase-coding region was recovered from vector LacZ-EF (a gift from Peter Kao, Stanford University, Palo Alto, CA) using digestion with SalI, Klenow fill-in, and Xbal cut and then cloned into pSecTagA previously digested with EcoRV and Xbal.

DNA preparation

Different DNA batches amplified at different times show heterogeneous properties in terms of the amount of DNA transcribed and subsequent protein synthesis (16). To decrease the possible effects of these fluctuations on DNA vaccination, each expression vector was amplified independently several times and eventually combined in one unique batch. To get rid of bacterial endotoxin, whose effect on DNA vaccination in a tolerogenic setting can be deleterious, DNA was purified with Triton X-114 (Sigma-Aldrich, St. Louis, MO) using digestion with XbaI, Klenow fill-in, and Xbal cut, and then cloned into pSecTagA previously digested with EcoRV and Xbal.

Mouse DNA vaccination and Ag sensitization

Female CBA/J (H-2k) mice were obtained from Harlan (AD Horst, The Netherlands) and reared in the animal facility in agreement with procedures submitted to the State Veterinary Office. In the prophylactic approach (Fig. 1A), mice were vaccinated at 8–10 wk of age with 100 μg of PLA2 (Latoxan, Rosans, France) given at 2-wk intervals (20). This indeed favored the induction of an anti-PLA2 IgE response and thus implied that the anaphylactic approach. Sera were collected at 2-wk intervals over 7 mo and kept at −80°C before analysis.

To ensure that DNA vectors were indeed functional, we performed the following control experiment: 2 wk after administration of pSecTagA-LacZ coding for the same conditions as those used for PL2_V, B16F10 constructs, the β-galactosidase substrate (Roche Molecular Biochemicals, Rotkreuz, Switzerland) applied locally yielded skin blue staining within 1 h, which was completely absent in mice not treated with DNA.

Measurement of serum Ab titers

Ab titers recovered at 2-wk intervals were measured by ELISA. The 96-well Nunc Maxisorp immunolates (Life Technologies, Basel, Switzerland) were coated for 1 h at 37°C with 50 μl of 5 μg/ml PLA2 (Latoxan) in coating solution (50 mM carbonate-bicarbonate (pH 9.6)). Nonspecific binding sites were blocked with 200 μl of PBS-0.05% Tween 20 (PBS-T)-1% BSA (Flika, Buchs, Switzerland) and incubated for 1 h at 37°C. After three washes with 300 μl of PBS-T, 50 μl of serial dilutions of murine serum in PBS-T-1% BSA was added and the plates were incubated overnight at 4°C. After washing as above, 50 μl of detection Ab in PBS-T-1% BSA, namely, 1) biotinylated goat Ab anti-mouse IgG diluted 1:3000 (Jackson ImmunoResearch, West Grove, PA), 2) biotinylated goat anti-mouse IgG1 diluted 1:3000 (Caltag,1)3) biotinylated goat Ab anti-mouse IgG2a diluted 1:3000 (Caltag), 4) biotinylated goat Ab anti-mouse IgG3 diluted 1:3000 (Caltag), 5) biotinylated rat Ab anti-mouse IgE diluted 1:250 (Pharmingen, San Diego, CA) were added to the appropriate wells and incubated for 1 h at 37°C. Plates were washed with PBS-T, and the Ab sandwich was revealed using 50 μl of Extravidin-alkaline phosphatase (Sigma) diluted 1:10,000. After incubation for 30 min at 37°C, the plates were washed six times with PBS-T before adding 50 μl of alkaline phosphatase substrate solution (1 M diethanolamine (Merck, Zurich, Switzerland), 1 mM MgCl2 and 1 mg/ml p-nitrophenylphosphate (Sigma)). Absorbance values were read at 405 nm and the Ab titers were determined as the reciprocal of the last dilution yielding absorbance values 2-fold higher than the preimmune serum.

PLA2 purification and detoxification for cell culture

To get rid of its intrinsic cytotoxicity on cell cultures, PLA2 in PBS was treated overnight at 37°C with a 100-fold molar excess of DTT (Fluka), then alkylated with a 1000-fold molar excess of N-ethylmaleimide (Fluka). After chemical modification, PLA2 was desalted on a Sephadex G-25 (Amersham Pharmacia Biotech, Zurich, Switzerland) column (1 cm × 30 cm) equilibrated and run in PBS.

Lymphocyte recovery, culture, and proliferation assay

Six months after the last sensitization with PLA2/alum (prophylactic protocol) or 5 mo after the last DNA administration (therapeutic protocol), mice were either directly sacrificed or challenged twice with 30 μg of native PLA2 and sacrificed 1 wk later. To assess the response against OVA, mice resistant to PLA2 were sensitized with OVA/alum and sacrificed 1 wk later. Spleen cells from individual animals were plated in a flat-bottom 96-well plate (Costar) Integra-Biosciences, Wallisellen, Switzerland) at further analysis. Mice sensitized with OVA received three to five doses of 1 μg of OVA combined with 1 mg of alum at 1-wk intervals.

In the therapeutic approach (Fig. 1B), 8- to 10-wk-old mice were first sensitized with six doses of PLA2/alum, then vaccinated with DNA constructs as for the prophylactic approach. Sera were collected at 2-wk intervals over 7 mo and kept at −80°C before analysis.

Table I. Sequences of 5′ sense and 3′ antisense primers used for cloning

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>5′ PLA2 (1)</td>
<td>5′-GACTCAAGCGGACGCGAGTCAGAAGATATAATATCCAGGAAGCTGTTATGG-3′</td>
</tr>
<tr>
<td>5′ PLA2 (2)</td>
<td>5′-GACTCAAGCGGACGCGAGTCAGAAGATATAATATCCAGGAAGCTGTTATGG-3′</td>
</tr>
<tr>
<td>5′ PLA2 (3)</td>
<td>5′-GACTCAAGCGGACGCGAGTCAGAAGATATAATATCCAGGAAGCTGTTATGG-3′</td>
</tr>
<tr>
<td>5′ PLA2 (4)</td>
<td>5′-GACTCAAGCGGACGCGAGTCAGAAGATATAATATCCAGGAAGCTGTTATGG-3′</td>
</tr>
<tr>
<td>5′ PLA2 (5)</td>
<td>5′-CGCGAATTTCGTTACTCAGGAGATGCAACATTCC-3′</td>
</tr>
<tr>
<td>5′ PLA2 (6)</td>
<td>5′-CGCGAATTTCGTTACTCAGGAGATGCAACATTCC-3′</td>
</tr>
<tr>
<td>5′ PLA2 (7)</td>
<td>5′-CGCGAATTTCGTTACTCAGGAGATGCAACATTCC-3′</td>
</tr>
<tr>
<td>5′ PLA2 (8)</td>
<td>5′-CGCGAATTTCGTTACTCAGGAGATGCAACATTCC-3′</td>
</tr>
<tr>
<td>5′ PLA2.FLAG</td>
<td>5′-ACTGGTGACGCGCCACCGGCGGCGGACTACAAGGACGAGCGATGACAAG-3′</td>
</tr>
</tbody>
</table>

* List of ODN used for the construction of PLA2 peptide expression vectors. 5′ sense primers (1-4): the sequence in italics carries the FLAG-coding region and that in bold encodes the eight first amino acids of PLA2. P1, P2, and P3, respectively; 3′ antisense primers (5-8): CCG, clamp for efficacious hybridization; wavy underlining, EcoRI site; bold underlining, STOP codon; bold characters, eight last codons for the C terminus of P1A, P2, and P3, respectively. 5′ PLA2.FLAG: the sequence in italics carries the coding region for the FLAG and the 5′ flanking site is shown underlined.
FIGURE 1. Experimental setting for the prophylactic and therapeutic approaches used. Arrows indicate the week at which mice were vaccinated with DNA, sensitized with PLA2/alum or OVA/alum, and challenged with PLA2. In the prophylactic approach (A), PLA2-specific Ab were measured from weeks 5–29, whereas in the therapeutic approach (B), Ab were measured from weeks 1–33, with the exception of week 15. Triggering of anaphylaxis, cytokine secretion, and lymphocyte proliferation were evaluated 2 wk after the last Ab titer measurement (PLA2) or 1 wk after the last OVA injection.

15–20 × 10^4 cells per 200 μl of DMEM (Life Technologies) complemented with 10% FCS (Life Technologies), 20 mM sodium pyruvate (Life Technologies), 2 mM l-glutamine (Life Technologies) and 5 × 10^{-3} M 2-ME, 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies). Alkylated PLA2 (10 μg/ml; see above) resuspended in plain DMEM was added for Ag-specific proliferation. Con A (Sigma) used at 2.5 μg/ml served as positive control. OVA used at 10 μg/ml and medium alone were used as negative controls. The cells were incubated for 4 days at 37°C and finally pulsed overnight with 1 μCi/well [3H]thymidine (Hartmann Analytic, Braunschweig, Germany). Cells were harvested and nuclear incorporation of radioactivity was measured in a scintillation beta counter (Topcount; Canberra Packard, Zurich, Switzerland). Proliferation responses were calculated as stimulation index dividing geometric mean Ag-stimulated cpm by background cpm.

Cytokine release assays

One million cells were incubated in 24-well plates in a final volume of 1 ml in the presence of PLA2. Con A, OVA, or plain medium. Supernatants were harvested at the indicated times, and cytokine concentrations were determined by ELISA using a combination of specific mAb according to the manufacturer’s protocol: IL-4: PharMingen, clones 11B11 and BVD6-24G2, 3-day-old supernatant; IFN-γ: PharMingen, clones R4-6A2 and XMG1.2, 2-day-old supernatant; and IL-10: PharMingen, clones JES5-2A5 and SXC-1, 3-day-old supernatant.

Statistical analysis

Comparison in cytokine secretion and T cell proliferation assays between groups of mice (see Figs. 3, 5, and 6) was evaluated by the paired Student t test using GraphPad Instat software Mac version 2.01 (San Diego, CA). SDs of Ab titers (see Figs. 2 and 4) were calculated using the function STDEVA from the Excel 98 application for Apple Macintosh (Cupertino, CA).

Results

DNA vectors coding for PLA2 and peptides are functional in vitro and in vivo

After cloning as described in Materials and Methods, Chinese hamster ovary cells were transfected with either one of the four expression vectors coding for the full-length PLA2 and derived peptides, and the production of proteins was assessed by dot blot and immunofluorescence (S. Jilek, unpublished observations). Proteins were found both intracellularly and in the culture medium, indicating that the vectors are able to govern transcription and translation in eukaryotic cells. As an additional control, pSecTagA-LacZ was administrated to CBA/J mice in the form of three doses of 100 μg at 1-wk intervals. Two weeks after the last application, the presence of the β-galactosidase enzyme at the site of administration was determined by injecting s.c. 150 μl of the substrate. Within 1 h, the skin of animals exposed to pSecTagA-LacZ turned blue, whereas control animals did not show any change in the skin complexion (S. Jilek, unpublished observations). With time, the staining was seen not only in the application zone but also in surrounding areas, indicating that the protein expressed by the cells at the site of injection diffused in the extracellular milieu. It took 24 h after substrate injection to observe complete disappearance of the coloration. Crucial to the subsequent interpretation of the data, these results indicate that all of the expression vectors are fully active in both in vitro and in vivo contexts.

PLA2-specific IgG1, IgG2a, IgG3, and IgE responses are modulated by prophylactic DNA vaccination

Either PLA2 or 45–60-aa-long peptides derived from PLA2 down-regulate specific IgE response in mice when given i.p. or via the nasal route (9, 10). However, relatively large amounts need to be administered repeatedly. To explore the potential of DNA vaccination to modulate the allergic immune response and prevent anaphylaxis, plasmids coding for PLA2, the PLA2-derived peptides devoid of possible recognition by IgE, and the empty vector were transfected via intradermal vaccination using three doses of 100 μg given at 1-wk intervals. Animals were then sensitized with 6 doses of PLA2/alum (see Materials and Methods), and the immunomodulation was analyzed first by measuring changes of the PLA2-specific Ig titers over a 6-mo time course (Fig. 2). Surprisingly, all DNA constructs containing or lacking PLA2-coding sequences led to very similar results. Prophylactic DNA treatment initially increased allergen-specific IgE production, as previously observed in conventional
immunotherapy (22). Starting at week 21, gene vaccination reduced IgE responses against PLA2 Ag for the duration of the analysis, in contrast to untreated animals exhibiting stable titers twice as high as those measured at week 17 (10; B. Corthézy, unpublished observations). The last measurement yielded average IgE titers even below those observed at the end of the sensitization phase. Consistent with the results, the weak burst seen for specific IgG1 Ab titers started decreasing 6 wk after sensitization, reflecting the inability of the vaccinated mouse to sustain production of this Th2-type Ig. An increase in specific IgG2a was obtained following PLA2/alum sensitization, suggesting a DNA vaccine-mediated skew toward a Th1 type of immune response (23). The rise in IgG2a titer seen with construct P1V known to contain a dominant T epitope (9) was largely due to two animals and was not reproduced using the PLA2V construct. When extending the analysis to the IgG3 isotype, a sustained titer rise was also detected, therefore suggesting at the Ab level that the immune response was actively biased to Th1 cytokines. Similar to IgE, IgG titers were two to four times higher with no DNA treatment and remained stable for the duration of the analysis. The very similar trend seen on the Ig pattern of either DNA constructs argues in favor of a dominance of the Th1-mediated response that was examined at the cellular and molecular levels.

Prophylactic DNA vaccination triggers a Th1 cytokine shift, modulates specific T cell responsiveness, and prevents anaphylaxis

Immune polarization was examined by the capacity of lymphocytes to secrete more IFN-γ (8) and IL-10 (24). In addition, the Th2 to Th1 switch is concomitantly accompanied by a marked drop in IL-4 synthesis (25, 26). T cell responses were thus assayed by proliferation of spleen cell cultures, since these latter yield the same cytokine profile as lymph node cells (9). T cells from mice immunized with either DNA plasmids recovered 30 wk after the last exposure to PLA2 produced up to 11-fold ($p < 0.0005$) more IFN-γ and 4.5-fold ($p < 0.0008$) more IL-10 than spleen cells from untreated, sensitized CBA/J mice kept in the same environment, but sensitized with PLA2/alum (Fig. 3A). The production of IL-4 was still detectable in the assay, but significantly reduced
in the PLA2-specific Ab responses could be measured at week 32.

The presence of medium alone or in the presence of 10 μg/ml OVA led to secretion when the spleen cells were cultivated in the absence of any Ag and cytokines were measured as described in Materials and Methods. B. Stimulatory index (S.I.) of splenocytes from mice challenged with PLAD (■) at week 31 or left unchallenged (□). Proliferation was performed in the presence of 10 μg/ml detoxified PLAD for 5 days. Splenocytes from untreated, nonsensitized mice yielded background levels (data not shown). C. Lack of anaphylactic reaction of vaccinated mice. All control mice (■) given 30 μg of PLAD i.p. died of anaphylactic shock, whereas mice treated with any DNA construct rapidly recovered from two identical PLAD doses.

FIGURE 3. Long-term analysis of immune markers of the DNA-vaccinated mice (prophylactic groups). A. Analysis of the cytokine production of splenocytes recovered from mice prophylactically vaccinated with various DNA constructs and sensitized with six i.p. injections of PLAD/alum. U. Untreated, sensitized mice. Mice were challenged with PLAD (■) at week 31 (recall challenge) or left unchallenged (□). Splenocytes were incubated with the indicated Ag and cytokines were measured as described in Materials and Methods. B. Stimulatory index (S.I.) of splenocytes from mice challenged with PLAD (■) at week 31 or left unchallenged (□). Proliferation was performed in the presence of 10 μg/ml detoxified PLAD for 5 days. Splenocytes from untreated, nonsensitized mice yielded background levels (data not shown). C. Lack of anaphylactic reaction of vaccinated mice. All control mice (■) given 30 μg of PLAD i.p. died of anaphylactic shock, whereas mice treated with any DNA construct rapidly recovered from two identical PLAD doses.

(p < 0.001) as compared with that seen using splenocytes isolated from untreated, sensitized mice. Together, this resulted in a pronounced rise in the Th1:Th2 ratio expected after skin surface DNA application. When mice were challenged twice with 30 μg of native PLAD followed by spleen cell extraction 1 wk later, we observed a 2-fold increase in the amount of IFN-γ and IL-10 (p < 0.005) after PLAD stimulation in vitro, with negligible changes in the IL-4 concentration (Fig. 3A). No triggering of IFN-γ and IL-10 secretion was observed when the spleen cells were cultivated in the presence of medium alone or in the presence of 10 μg/ml OVA, although the three cytokines were still produced to a similar extent as before PLAD challenge (Fig. 3A). Together, the data indicate that DNA vaccination has prompted the T cell response to evolve toward a Th1 profile and that subsequent sensitization with PLAD/alum allows an Ag-specific memory response to be preserved for up to 7 mo. Moreover, after PLAD challenge, no significant change in the PLAD-specific Ab responses could be measured at week 32 (Fig. 2), suggesting Th1-polarized memory responses.

No T cell proliferation could be detected using the spleen of DNA-vaccinated mice left for 7 mo in the absence of any PLAD challenge (Fig. 3B) or the spleen of untreated, nonsensitized mice (S. Jilek, unpublished observations). In contrast, two doses of 30 μg of PLAD before spleen cell recovery made them capable of proliferating specifically (p < 0.004) in the presence of PLAD in vitro (Fig. 3B). Consistent with the increase in IFN-γ and IL-10 expression, this suggests that an active immune deviation mediated by Th1 cytokines is actually taking place, coupled with a state of unresponsiveness in the absence of Ag challenge. Furthermore, the physiological relevance of 1) the resistance to IgE induction, 2) the production of cytokines of the Th1 type, and 3) the capability to proliferate upon specific Ag stimulation is demonstrated by the 100% survival rate of DNA-vaccinated mice exposed to one and two injections of 30 μg of PLAD (Fig. 3C). The protection against anaphylaxis was dependent on the prophylactic treatment, as sensitized, nonvaccinated mice all underwent an immediate drastic temperature drop and died within 30 min after the first administration of challenging PLAD (Fig. 3C).

Therapeutic DNA vaccination prevents PLA2-specific IgE response, enhances Th1 cytokine secretion, and partially blocks anaphylaxis

Given the high effectiveness of DNA vaccination both in terms of preventing the development of an IgE-mediated response and inducing long-lived protective immune memory, we examined the possibility of down-regulating the course of an established allergic response. Mice were vaccinated intradermally using three doses of 100 μg of DNA constructs given at 1-wk intervals. The same markers of the immune response as for the prophylactic protocol were analyzed. Following PLAD sensitization, the PLAD-specific Ig Ab titers raised during the first 4 wk with a kinetics lacking the lag phase seen in the prophylactic approach (Fig. 2). DNA vaccination led to the reduction of IgE and IgG1, with a concomitant increase of IgG2a and IgG3, yet to a less marked, but appreciable, extent than in mice treated with the prophylactic approach (Fig. 4). Mice not treated with the DNA vaccine kept exhibiting stable IgG and IgE titers (Ref. 9; S. Jilek, unpublished observations). Once again, the presence of PLAD-coding sequences on the DNA vector did not significantly affect the fluctuations in Ig titer. This suggests that a pre-established Th2 allergic response can be redirected by Ag-independent DNA therapy favoring secretion of Ig isotypes controlled by Th1 cytokines. Consistent with this, no significant change in the PLAD-specific Ab responses could be measured at week 36 following PLAD challenge (Fig. 4).

T cells recovered from spleen 5 mo after the last exposure to PLAD/alum were found to be able to secrete IFN-γ and IL-10 to a comparable extent as T cells obtained from mice in the prophylactic groups, well above (p < 0.0003) the level detected in untreated, sensitized mice (Fig. 5A). Challenge with native PLAD led to a roughly two times higher release of IFN-γ and IL-10 by PLAD-
stimulated T cells \( (p < 0.008) \). For the IL-4 production, we observed a 3-fold drop \( (p < 0.01) \) as compared with that measured with cells from untreated, sensitized mice, which reflected the observation made at the Ab level. Before PLA2 challenge, no Ag-specific proliferation could be detected, whereas a 2- to 3-fold increase \( (p < 0.007) \) in the stimulation index was obtained after PLA2 challenge (Fig. 5B). Similar to what we concluded from the prophylactic protocol, PLA2 challenge reactivated quiescent T cells to secrete IFN-\( \gamma \) (27) and maintain a Th1 milieu attenuating the allergy-oriented Th2 immune response. In contrast to the full protection against anaphylaxis seen with mice treated prophylactically, 70% of the mice showed long-lasting immobility, of which half did not recover and eventually died (Fig. 5C). Interestingly, mice showing no sign of anaphylaxis after the first challenge could bear a second challenge with 30 \( \mu \)g of PLA2. No significant correlation with the remaining IgE titer could be drawn, a situation also encountered in conventional immunotherapy involving human patients.

The Th1 milieu resulting from DNA vaccination inhibits primary and recall responses against OVA used as a control Ag

Our data have shown that the Ag-coding sequences are not essential in the DNA plasmid during the initial phase of Th2 to Th1 deviation, and that the Th1 milieu itself is sufficient to take control of the allergic reaction induced by Ag administration before or after gene therapy. We therefore reasoned that the unexpected observation we made for the PLA2 protein would benefit from its confirmation using another Ag protein and thus substantiate the dominant Th1 bystander effect revealed in this study. Two series of control experiments were conducted with OVA. First, mice were vaccinated three times with empty pSec-TagA at 1-wk intervals, and 2 wk after the last DNA application were sensitized five times with OVA/alum at 2-wk intervals. OVA-specific IgE measured in the serum of vaccinated mice 2 wk after the last OVA/alum injection were reduced by a factor of 3.5 as compared with untreated, sensitized mice \( (p < 0.002) \), while IgG2a and IgG3 levels were
similar to the prophylactic approach with the PLA2 Ag, the cytokine response to the PLA2 prophylactic protocol were sensitized 6 mo after the last DNA application with three i.p. applications of OVA/alum. Untreated, sensitized mice. Mice were challenged with PLA2 (M) at week 35 (recall challenge) or left unchallenged (□). Splenocytes were incubated with the indicated Ag and cytokines were measured as described in Materials and Methods. B, Stimulatory index (S. I.) of splenocytes from mice challenged with PLA2 (M) at week 35 or left unchallenged (□). Proliferation was performed in the presence of 10 μg/ml detoxified PLA2 for 5 days. Splenocytes from nonsensitized, untreated mice yielded background levels (data not shown). C, Lack of anaphylactic reaction of vaccinated mice. All control mice (−) given 30 μg of PLA2 i.p. died of anaphylactic shock, a phenomenon limited to one-third of the mice treated with any DNA construct challenged with one or two PLA2 doses.

FIGURE 5. Long-term analysis of immune markers of the DNA-vaccinated mice (therapeutic groups). A, Analysis of the cytokine production of splenocytes recovered from mice therapeutically vaccinated with various DNA constructs after sensitization with six i.p. injections of PLA2/alum. Untreated, sensitized mice. Mice were challenged with PLA2 (M) at week 35 (recall challenge) or left unchallenged (□). Splenocytes were incubated with the indicated Ag and cytokines were measured as described in Materials and Methods. B, Stimulatory index (S. I.) of splenocytes from mice challenged with PLA2 (M) at week 35 or left unchallenged (□). Proliferation was performed in the presence of 10 μg/ml detoxified PLA2 for 5 days. Splenocytes from nonsensitized, untreated mice yielded background levels (data not shown). C, Lack of anaphylactic reaction of vaccinated mice. All control mice (−) given 30 μg of PLA2 i.p. died of anaphylactic shock, a phenomenon limited to one-third of the mice treated with any DNA construct challenged with one or two PLA2 doses.

Discussion
Intradermal gene vaccination of mice induces Ag-specific Th1 cells that secrete high levels of IFN-γ and stimulates production of Abs of the IgG2a and IgG3 isotypes (28). Our data show that the effect of DNA immunization is dominant, since it prevents the subsequent induction by PLA2/alum of either an IgE Ab response or activation of Th2 cells producing IL-4; it can also reduce a pre-existing allergen-specific IgE response. Whether this is accompanied by the down-regulation of basophils and mast cell activation by IFN-γ remains to be determined (29, 30). The novelty of our data resides in the observation that the allergen does not need to be codelivered by the DNA plasmid, but its mere presence as an exogenously delivered protein after gene vaccination promotes naïve CD4+ T lymphocyte differentiation toward Th1 cells, leading to a second burst of IFN-γ production in an allergen-dependent fashion. When gene vaccination was followed by Ag delivery, reversal of Th2-type responses was observed, underlining the plasticity of the system in vivo and the dominant effect of DNA-induced Th1 cytokines over a pre-existing Th2 pattern. Specifically, we demonstrate herein that PLA2-independent gene vaccination down-regulates an ongoing Th2 response in favor of a Th1 profile in both a prophylactic and therapeutic approach, ultimately resulting in prevention of anaphylaxis in CBA/J mice.

In other studies describing the preventive action of DNA immunotherapy in allergy models, the properties of empty DNA constructs were not evaluated (18) or, if so, were examined in other mouse and rat strains and more importantly after i.m. injection (14, 16, 31), resulting in tissue injury and inflammation favoring stronger local immune response (32). Furthermore, in contrast to our own experimental setting, DNA preparations were not devoid of bacterial endotoxin. It remains to determine whether this is crucial to the outcome of the immune response. DNA plasmids administered intradermally have been followed through in BALB/c mice, and tissue spreading beyond regional lymph nodes has been established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35). Consistently, our data stress that the cytokine milieu before Ag challenge is established (33). However, RT-PCR analysis of various tissues after injection of DNA revealed no Ag expression (34), raising the question of the identity of the APCs involved and the necessity of presentation at the time of DNA “reading” by the host (35).
same observations as those reported in this work. In this respect, birch pollen isoforms coded by DNA plasmids triggered either a strong Ag-specific Th1 response (Betv1a), or resulted in no proliferation nor cytokine release (Betv1d) in the absence of sensitization (38).

Our results point to the fact that immunotherapy can be accomplished by DNA lacking any coding sequences for the allergen under study. What therefore are the possible mechanisms explaining the protective function of Ag-independent gene vaccination? A clue to this puzzling question might come from the concept that DNA used for vaccination can be divided into two units consisting of a transcriptional unit directing Ag synthesis and an adjuvant/mitogen unit in the plasmid backbone acting on cells of the innate branch of the immune system and trigger an initial burst of IFN-γ in an allergen-independent manner.

The pSecTagA used in this study comprises as many as 23 ISS, and thus it makes sense that it can stimulate the innate immune system to create a cytokine milieu that favors the generation of a Th1-biased response to the Ag. The in vivo adjuvant activity of ISS before Ag administration is referred to as “prepriming” (44, 45). Consistent with our observations that ISS per se can suppress markers of an allergic reaction, prevention of allergic lung inflammation in a mouse model of asthma was reduced by intratracheal administration of CpG ODN alone before allergen challenge (46). The CpG ODN increased the ratio of IFN-γ:IL-4, diminished eosinophilia, and reduced Ag-specific IgE-producing cells. Similar to our data, a sustained Th1 memory response to the recall Ag was detected for at least 6 wk after ODN administration (46). This is attributed to increased IFN-γ concentrations and decreased IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluids (47). In the case of allergic hyperresponsiveness, ISS should hence be seen as dominant negative modulators (48) due to their intrinsic capacity to positively influence the development of memory Th1 cells.

Our data show that the plasmid DNA does not need to contain the coding sequence for the allergen to down-regulate the allergic response, implying that Th1 deviation by itself is sufficient to protect against subsequent challenge with the allergen protein. This is indeed reflected by the OVA sensitization of mice, which resulted in the preserved secretion of IFN-γ in the culture supernatant of the same cells in vitro (Fig. 6). Likewise, P2V and P3V lacking T epitope in the coding sequences they carry are as good as EV in preventing production of markers of allergic and Th2 immune responses. In contrast to P2 and P3 peptides administered as such (9), IgE Ab titers to OVA injected 6 mo after the last DNA application (EV or PLA2V) were reduced as compared with nonvaccinated animals; this suggests that the prevalence of the Th1 response was preserved as marked by sustained IFN-γ production and can be seen as what we call a cytokine milieu memory effect.

DNA, or ISS thereof, might confer to APC the capacity to present Ag to T cells bathed within a Th1-biased cytokine milieu and therefore prime the synthesis of IgG2a and IgG3 Ab preferentially.

Remarkably, our data demonstrate that skin surface scrapping of DNA plasmids containing ISS motifs rapidly stimulating the host to mount a Th1-dominated innate response is operative in both the prophylactic and therapeutic settings. In addition, the documented absence of local inflammation after intradermal DNA immunization might preclude the induction of costimulatory molecules including CD86, CD40 (49) on APCs (most likely skin Langerhans cells), and maintain these latter in a status of presentation inducing tolerance (50). Another advantage of DNA resides in its capacity to “survive” for long periods in the body (51) and thus function as some sort of an adjuvant reservoir favoring Th1 type cytokines. We believe this might account for the long-term memory seen in this study and also explain the rapid burst in the production of IgG2a and IgG3 directed against sensitizing doses of PLA2 in the prophylactic or therapeutic approaches. Interestingly, the maintenance of IL-4 production suggests that the vaccinated organism is not fully impaired in its potential to mount a Th2-type immune response. This has implications in protection against parasites for example (52).

The increase in PLA2-specific IgG2a and IgG3 Abs observed in mice after prophylactic and therapeutic gene vaccination might block serum-facilitated allergen presentation (53), thus mimicking the protective function of IgG4 in humans (54). The role of IgG1 Abs blocking the Ag-IgE binding through recognition of similar
epitopes (55) suggests that the Th1/Th2 dichotomy reflected by production of IgG isotypes might represent an oversimplification when seeking markers of immunomodulation. However, the contribution of various maternal Ag-specific IgG1 and IgG2b Abs to suppression of the IgE immune response to bee venom PL-A2 in CBA/J mice offspring argues in favor of the possible role of such Ab (21). The relevance of serum-facilitating allergen presentation is further acknowledged by the recent report of van Neerwen et al. (56) who were able to demonstrate its effectiveness in patients allergic to birch pollen.

In summary, this is the first report that Ag-independent and prolonged suppression of an allergic reaction is modulated by DNA vaccination. Intradermal administration of DNA lacking any coding sequence for the Ag preferentially 1 stimulated the production of Th1 cytokines, 2 suppressed Ag-specific IgE, 3 triggered Ag-specific IgG2a and IgG3, 4) and blocked or reduced anaphylaxis after prophylactic and therapeutic treatment, respectively. The data presented in this study should prompt the further examination of DNA vaccination in the context of the expected effects (Th switch, Ab production, cellular responses, induction of) on the immune system in terms of Ag mobilization, the sites and means of delivery, the amount and nature of DNA, as well as the target organisms (31). It is conceivable that the administration of ISS with a given allergen could be used in atopic persons to modify the Th2-oriented allergen-specific response. In addition, in the animal experiments that mirror the situation in humans, it is now possible to analyze whether the deficit in Th1-stimulating microbial infections encountered in developed countries (and suggested as one possible cause for the increase of atopic diseases [57–59]) might be compensated for by treatment with Ag-independent, ODN-mediated therapy.

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References