

Modulation of allergic responses in mice by using biodegradable poly(lactide-co-glycolide) microspheres

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Background: Biodegradable poly(lactide-co-glycolide) (PLGA) microspheres are a promising carrier for vaccine delivery capable of maturing antigen-presenting cells to stimulate T-cell-mediated immune responses. However, the potential of microspheres to downregulate an allergic response *in vivo* is unknown.

Objective: The aim of this study was to determine whether microspheres could potentiate DNA vaccination against allergy and to evaluate the immunomodulatory properties of microspheres alone.

Methods: Mice were treated prophylactically with DNA-loaded plain PLGA microspheres before sensitization with phospholipase A2 (PLA2), the major allergen of bee venom. PLA2-specific IgG1, IgG2a, IgE in serum were measured for 8.5 months, and splenocyte proliferative responses and cytokine profiles were determined. Protection against anaphylaxis was evaluated after injection of an otherwise lethal dose of PLA2. **Results:** Phospholipase A2-specific IgG1 and IgG2a production turned out to be 2 times higher using cationic microspheres compared with anionic microspheres, but was not influenced by the presence of DNA. In contrast, reduction in IgE production and T-cell hyporesponsiveness were observed with all microsphere formulations. Recall challenge with PLA2 triggered combined expression of both IL-4 and IFN- γ , together with sustained expression of IL-10 that can explain the protective effect against anaphylaxis.

Conclusion: Our data suggest a dual mechanism that does initially rely on a T_H2 to T_H1 immune deviation and then on IL-10-mediated suppression. This is the first physiological demonstration that plain PLGA microspheres can induce tolerance in mice for as long as 6 months postsensitization. (J Allergy Clin Immunol 2004;114:943-50.)

Key words: Allergy, microspheres, T_H1/T_H2 responses, immunomodulation

Abbreviations used

DC: Dendritic cell
EV: Empty vector
PLGA: Poly(lactide-co-glycolide)
PLA2: Phospholipase A2
PLA2V: Phospholipase A2-coding vector
SI: Stimulatory index

In developed countries, about 20% to 30% of the population has allergy.¹ The exposure to normally harmless airborne or food antigen can lead to hay fever, urticaria, eczema, or asthma. Another source of potential allergens is represented by insect venoms. This kind of allergy is potentially more dangerous because a single bee sting can induce an anaphylactic shock leading to death. Currently, specific antigen immunotherapy based on injection of increasing doses of allergen is used to desensitize patients. During specific antigen immunotherapy, IL-4 and IL-5 production decreases, IFN- γ production increases,²⁻⁴ and a state of hyporesponsiveness is induced at the end of the treatment.⁵ In contrast with classical vaccination, specific antigen immunotherapy is a long-lasting (3-5 years) process for the patient.⁶ As an alternative to immunomodulating the allergic responses, DNA vaccination was proven to skew the T-cell response toward a T_H1 profile and trigger memory.^{7,8}

Biodegradable and biocompatible poly(lactide-co-glycolide) (PLGA) microspheres of defined composition, size, and surface property permit delivery of protein/polypeptide antigen to an organism in a continuous or pulsatile manner, thus opening the way for single-injection vaccines.⁹⁻¹¹ PLGA microspheres are targeted to dendritic cells (DCs) or macrophages by unspecific phagocytosis as demonstrated *in vitro*¹² and *in vivo*.^{13,14} This triggers maturation of DCs,^{15,16} which are able to process and present the encapsulated antigen on MHC class I^{17,18} and MHC class II¹⁷ molecules, ensuring optimal presentation of antigen in secondary lymphoid tissues.¹⁹ Encapsulated or surface-adsorbed DNA extracted from microspheres was shown to be bioactive and able to trigger transfection of cells *in vitro*.²⁰⁻²² Accordingly, encapsulation of DNA in PLGA microspheres and adsorption of DNA on the surface of cationic microspheres containing

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TABLE I. Various formulations of microspheres, microspheres/DNA, and DNA alone administered to mice

Nomenclature of groups of mice	Polymer type*	Amount of DNA in microspheres ($\mu\text{g}/\text{mg}$)	Total amount of DNA administered (μg)
PLGA	PLGA	—	0
PLGA/EV (enc)	PLGA	3-5	45-75
PLGA/PLA2V (enc)	PLGA	3-5	45-75
PLGA/PEI	PLGA, PEI	—	0
PLGA/PEI/EV (co)	PLGA, PEI	25-35	375-525
PLGA/PEI/PLA2V (co)	PLGA, PEI	25-35	375-525
EV	—	—	300
PLA2V	—	—	300

co, Adsorption by surface coating; enc, encapsulation.

*Microspheres containing polyethylenimine (PEI) display a positive surface charge, which is maintained on DNA adsorption, whereas all other microspheres display a negative surface charge.

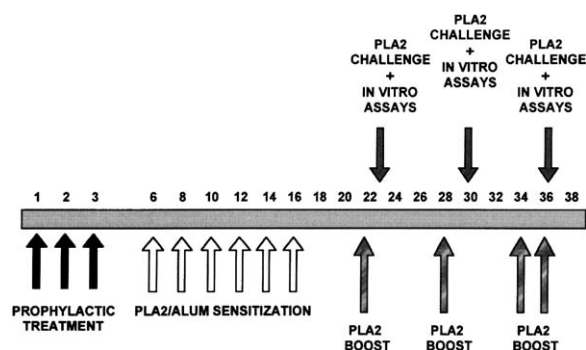


FIG 1. Experimental setting used for the vaccination approach. Black arrows indicate the week at which the mice received the different microsphere (MS) formulations. White arrows indicate the week at which the mice were sensitized with 0.1 μg PLA2 in alum. Light grey arrows mark additional boosts performed with 0.1 μg PLA2 in alum before challenge with 30 μg anaphylactogenic PLA2 (dark grey arrows). Mice were killed after challenge, and *in vitro* assays composed of T-cell proliferation and cytokine measurements were performed.

polyethylenimine¹⁶ were able to induce potent immune responses *in vivo*.²³⁻²⁵

In this study, the immunomodulatory capacity of DNA-loaded and plain biodegradable PLGA microspheres was tested in a mouse model of bee venom phospholipase A2 (PLA2) allergy.⁸ After injection of PLGA microspheres in mice, reduced IgG1 and IgG2a titers were found for anionic microspheres compared with cationic microspheres or DNA alone, whereas changes in IgE were similar in all groups. After allergen sensitization starting at week 24, we observed both IL-4 and IFN- γ expression in splenocytes, together with sustained IL-10. In good correlation with observed downregulation of T-cell proliferation, 50 mice of 54 were protected against lethal PLA2 challenge. We conclude that administration of microspheres, although not capable of fully reversing a T_H2 response in favor of a T_H1 bias, triggers transient protection against anaphylaxis in mice, underlying a mechanism implying IL-10 synthesis and suppressed antigen-specific T-cell responsiveness.

METHODS

Chemicals

Poly(D,L-lactide-co-glycolide) (PLGA, Resomer RG502H, ratio lactide:glycolide 50:50, molecular weight 13,700) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Polyethylenimine, branched, molecular weight 600,000 to 1,000,000, was obtained from Fluka (Buchs, Switzerland).

Construction of expression vector

The expression vector used was pSecTagA (empty vector [EV]; Invitrogen, Breda, The Netherlands). Expression vector pSecTagA-PLA2 (PLA2V) coding for the whole bee venom PLA2 was constructed from pSecTagA as previously described.⁸

DNA preparation

To decrease the possible effects of using different DNA batches on vaccination,²⁶ plasmids were amplified independently several times and combined in 1 unique batch. To remove bacterial endotoxin, DNA was purified with Triton X-114 (Sigma, Buchs, Switzerland), as reported.⁸ DNA was dissolved at a concentration of 5 mg/mL in sterile endotoxin-free water and stored at -20°C .

Preparation and characterization of microspheres

Various formulations of biodegradable PLGA microspheres (size range, 1-10 μm)¹² were prepared by spray-drying as described elsewhere (Table I).^{20,27} DNA loading onto cationic microspheres was performed as described²² and assessed by photometric analysis (OD 260 nm) of the remaining DNA postadsorption. DNA loading of anionic and cationic microspheres formulations were 3 to 5 μg and 25 to 35 μg per milligram microspheres, respectively (Table I).

Administration of microspheres formulations to mice and antigen sensitization

Female CBA/J mice were obtained from Harlan (AD Horst, The Netherlands), reared in the animal facility, and subjected to experimental procedures approved by the State Veterinary Office. Mice were administered subcutaneously at 6 to 8 weeks of age with 5 mg of the various microspheres formulations in 100 μL 0.25% methocel in nanopure water once a week for 3 consecutive weeks (Fig 1, Table I). In control experiments, 3 times 100 μg naked PLA2V was applied as previously described at the base of the tail.⁸ Three weeks later, mice were sensitized subcutaneously with 6 doses of 0.1 μg PLA2 (Latexan, Valence, France) combined with 1 mg alum (Sigma) given

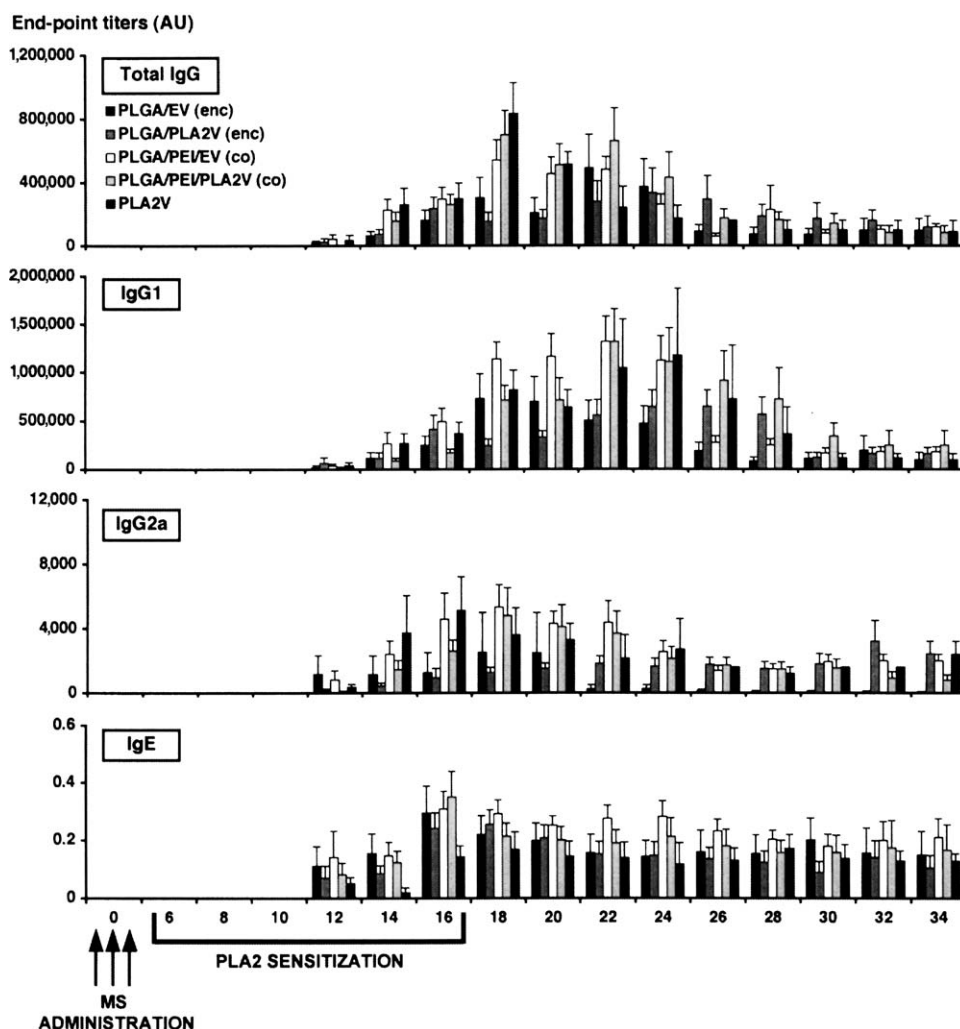


FIG 2. Antibody production in mice after administration of DNA-loaded microspheres (MS) and sensitization with PLA2/alum. Data are expressed as serum titers (IgG isotypes) or OD using 1:40 dilution (IgE) and represent the means of 11 mice (weeks 1-23), 8 mice (weeks 24-30), and 4 mice (weeks 31-36). Error bars represent SEM. AU, Arbitrary units; co, adsorption; enc, encapsulation; PEI, polyethylenimine.

at 2-week intervals.⁸ Over a period of 8.5 months, mice were bled every 2 weeks, and sera were kept at -80°C until further analysis.

Measurement of serum antibody titers

Antibody titers recovered at 2-week intervals were measured by ELISA as described by Jilek et al⁸ and represent the reciprocal of the last dilution yielding absorbance values 2-fold higher than the preimmune serum.

Phospholipase A2 challenge in mice and anaphylactic reactions

Four weeks after the last sensitization with PLA2/alum, mice were boosted with $0.1\text{ }\mu\text{g}$ PLA2/alum at 3 occasions (Fig 1). Three days later, mice were challenged intraperitoneally once with $30\text{ }\mu\text{g}$ native PLA2 in PBS, a dose that kills 100% of mice within 30 minutes in the absence of pretreatment.⁴ Subsequent anaphylaxis was examined on the basis of symptoms including scratching, agitation, spasm, immobility, inclination to gathering, increased breathing, and, in some cases, death.

Lymphocyte recovery, culture, and proliferation assay

After PLA2 challenge and anaphylactic reactions analysis, mice were killed and splenocyte proliferation performed as described by Jilek et al.⁸ Proliferation responses were calculated as stimulation index (SI) dividing geometric mean of antigen-stimulated counts per minute by background counts per minute. To analyze cytokine expression, splenocytes were incubated for 48 hours in the presence of $10\text{ }\mu\text{g/mL}$ detoxified PLA2 (specific antigen), $2\text{ }\mu\text{g/mL}$ Concanavalin A (control for cell viability), or plain medium (background level). Because PLA2 is toxic to most cells in culture, reduction-alkylation⁴ guarantees detoxification essential for valid proliferation assay.

Real-time RT-PCR

Total RNA from splenocytes was isolated by using the Chomczynski and Sacchi²⁸ method. Reverse transcription was performed by using 50 ng total RNA and $2.5\text{ }\mu\text{mol/L}$ primer dT₁₆ (Applied Biosystems, Rotkreuz, Switzerland). Specific pairs of primers for IL-4 (sense: $5'-\text{GGCATTTTGAACGAGGTCA}-3'$;

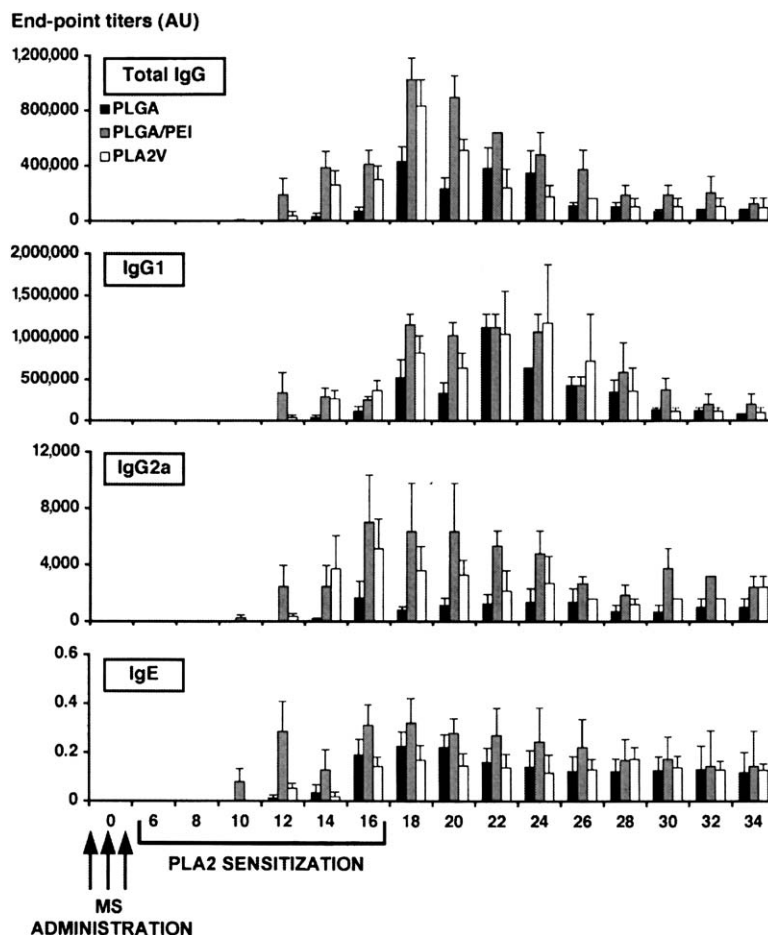


FIG 3. Antibody production in mice after administration of plain microspheres (MS) and sensitization with PLA2/alum. Blood samples were withdrawn every other week, and antibody production was analyzed. Data are expressed as serum titers (IgG isotypes) or OD using 1:40 dilution (IgE) and represent the means of 5 mice (weeks 1-23), 3 mice (weeks 24-30), and 2 mice (weeks 31-36). Error bars represent SEM. AU, Arbitrary units; PEI, polyethylenimine.

antisense: 5'-AGGACGTTTGGCACATCCAT-3'), IFN- γ (sense: 5'-ACAATGAACGCTACACACTGCAT-3'; antisense: 5'-TGGC-AGTAACAGCCAGAAACA-3'), and IL-10 (sense: 5'-CCTTC-AGCCAGGTGAAGAC-3'; antisense: 5'-GGAGTCGGT-TAGC-AGTATG-3') were mixed with SYBR green PCR master mix (Applied Biosystems), and 2 μ L of the previous reverse transcription volume containing cDNA were added. Standardization was performed by using primers targeting cyclophilin expression (sense: 5'-ACCCACCGTGTCTTCGA-3'; antisense: 5'-GGAACTTTGTCTGCAAACAG-3'). The real-time PCR reactions were performed by using an ABI Prism Sequence Detection System 7700 (Applied Biosystems). PCR conditions were as follows: T_{denaturation}: 95°C, 15 seconds; T_{annealing and extension}: 60°C, 1 minute; 40 cycles. A post-PCR dissociation curve was run to assess the specificity of amplified sequences. Results were analyzed by using the ABI Sequence Detection Systems 1.9.1 and Dissociation Curve software (Applied Biosystems). To reflect directly the differences in the expression of IL-4, IFN- γ , and IL-10, data were expressed as $100 \times (1/[C_{\text{cytokine}} - C_{\text{cyclophilin}}])$, where Ct represents the cycle number where the SYBR green fluorescence crossed the threshold value set at 0.2. Ratios of IFN- γ :IL-4 lower than 1 indicated a T_{H2} profile, and a T_{H1} bias when higher than 1.

Statistical analysis

SEMs of antibody titers, SI, and cytokines production (Figs 2-4) were calculated by using the function STDEVA from the Excel X application for Apple Macintosh (Cupertino, Calif). For antibody production, proliferation assays, and cytokine expression, statistical comparison was performed by using the nonpaired Mann-Whitney test performed with the statistic software Instat (GraphPad Software, San Diego, Calif).

RESULTS

Phospholipase A2-specific IgG, IgG1, IgG2a, and IgE responses are modulated by administration of DNA-loaded microspheres

We hypothesized that microspheres can enhance the bioavailability of plasmid DNA *in vivo*, providing long-lasting delivery in the tissues. To assess the specific effect of microspheres between experimental groups, we

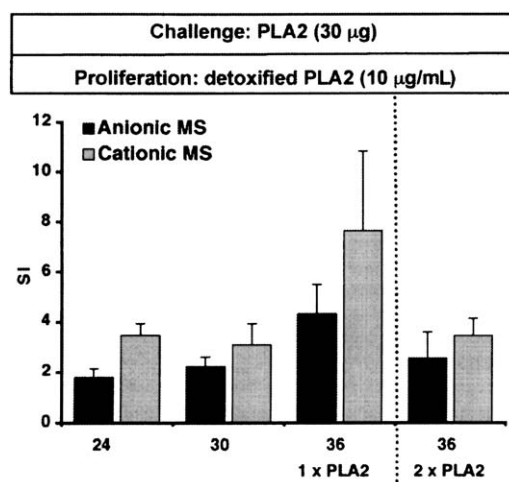


FIG 4. Stimulatory index (SI) of splenocytes from mice sacrificed at 3 time points after PLA2/alum sensitization. Mice were boosted once with PLA2/alum 3 days before sacrifice at weeks 24 and 30 and either once or twice at 1-week intervals at week 36. Data are expressed as the means of 5 mice (weeks 24 and 36) and 8 mice (week 30) for anionic microsphere (MS) formulations and as means of 5 mice (weeks 24 and 36) and 9 mice (week 30) for cationic MS formulations. Error bars represent SEM.

administered the same amount of cationic or anionic microspheres containing different quantities of associated DNA. Both EV and PLA2V plasmid DNA were either encapsulated in anionic microspheres or adsorbed onto the surface of cationic microspheres, resulting in a loading averaging between 4 μ g (encapsulation) and 30 μ g (adsorption; Table I). After prophylactic administration of 3×5 mg of DNA-loaded microspheres, mice were sensitized with 6 doses of PLA2/alum as described in Fig 1. The production of IgE and IgG1 used as a T_H2 marker, and IgG2a reflecting a T_H1 bias, was examined by measuring the changes in PLA2-specific Ig titers over a 8.5-month time course (Fig 2).

Although microspheres formulations led to an apparent increased production of IgE compared with DNA alone, this result was not statistically significant ($P > .05$). Over time, production of IgE was blocked and returned to the level measured at the end of PLA2 sensitization. Anionic DNA-loaded microspheres yielded IgG1 titers 2 times lower than their cationic counterpart during weeks 18 to 22 ($.02 < P < .04$). In control experiments, plasmid DNA induced a drop in IgG1 titers after week 24, whereas a delay of 4 to 6 weeks was detected with PLA2V-loaded microspheres. An increase in IgG2a titers was obtained after treatment with microspheres, with again a more acute rise in antibody titers when cationic microspheres were administered. For both microsphere formulations, the peak of IgG2a antibody preceded that of IgG1, suggesting an initial bias toward a T_H1 type of response. In contrast with the IgG1 antibody response, IgG2a production remained sustained for as long as 4 months after the last PLA2 sensitization. Thus, DNA-loaded microspheres preferentially favor the maintenance of T_H1 -type IgG2a and do not reinforce the effect of plasmid DNA. This is particularly true for cationic microspheres used to deliver

150 μ g DNA per administration compared with 100 μ g of DNA alone ($P > .05$).

Cationic and anionic microspheres per se modulate PLA2-specific antibody production

This prompted us to analyze the potential of plain cationic and anionic microspheres to modulate the allergic response triggered by PLA2 sensitization over a 34-week time course. After sensitization, mice treated with plain microspheres showed increased production of PLA2-specific IgE, which in time returned to levels reached at the end of the sensitization period (Fig 3). The IgG1 and IgG2a patterns looked very similar to those observed with DNA-loaded microspheres. Again, cationic microspheres favored the production of T_H1 -type IgG2a during weeks 18 to 22 ($.02 < P < .03$), peaking with a faster kinetics as compared with that of IgG1 appearance. The antibody titers lay in the range obtained after vaccination with DNA alone (Fig 3). We conclude that microspheres by themselves exert immunomodulatory properties as reflected by the immune polarization assessed by the IgG2a:IgG1 antibody ratio.

Administration of microspheres protected mice against anaphylaxis induced by PLA2 challenge

To determine whether injection of microspheres protected against anaphylactic reactions, mice were challenged with a lethal dose of PLA2 (30 μ g) at 3 different time points after administration of microspheres (weeks 24, 30, and 36). Mice from the control plasmid DNA group were challenged at weeks 24 and 36 only. At weeks 24 and 30, mice were boosted once with 0.1 μ g PLA2 in

TABLE II. Cytokine expression of splenocytes from mice sacrificed at 3 different time points after PLA2/alum sensitization and stimulated with PLA2

Cytokines	Experimental groups	Week 24	Week 30	Week 36
IL-4	Anionic microspheres	11.14* $\pm$ 1.25†	12.76 $\pm$ 2.18	19.94 $\pm$ 0.54
	Cationic microspheres	12.62 $\pm$ 0.82	12.49 $\pm$ 0.39	19.04 $\pm$ 0.79
	PLA2V	11.88	ND	16.80
IFN- γ	Anionic microspheres	14.67 $\pm$ 1.58	13.46 $\pm$ 2.50	23.70 $\pm$ 2.82
	Cationic microspheres	17.54 $\pm$ 0.70	12.93 $\pm$ 1.94	22.63 $\pm$ 6.04
	PLA2V	13.60	ND	27.51
IL-10	Anionic microspheres	10.54 $\pm$ 1.09	9.99 $\pm$ 0.89	9.54 $\pm$ 0.03
	Cationic microspheres	8.19 $\pm$ 4.13	10.96 $\pm$ 0.47	10.52 $\pm$ 0.39
	PLA2V	10.52	ND	10.04

*Expression of IL-4, IFN- γ , and IL-10 was analyzed by real-time RT-PCR and is expressed as $100 \times (1/\Delta Ct)$. Data are expressed as mean of 3 to 4 mice per microsphere formulation.

†SEM.

alum 3 days before challenge. At week 36, mice were boosted either once or twice at 1-week intervals with 0.1 μ g PLA2 in alum (week 36). Altogether, only 4 of 54 mice injected with microspheres formulations died of anaphylactic reactions. Moreover, at each time point (weeks 24, 30, and 36), symptoms as defined in Methods were similar between experimental groups. However, at week 36, more acute symptoms were seen in all mice, yet all but 2 recovered within 1 hour (data not shown). This is in agreement with the high survival rate achieved for mice vaccinated with PLA2V plasmid DNA (data not shown).⁸ We conclude that mice were protected against an otherwise lethal challenge of PLA2, irrespective of the treatment received.

Splenocyte proliferation was not dependent of the surface charge of the microspheres

Splenocyte proliferation assays were performed as described in Methods to determine whether the administration of microspheres formulations modulated the degree of activation of T cells. In all microsphere-treated groups, SIs ranging between 2 and 8 were obtained on stimulation with PLA2 (Fig 4), thus reflecting active immunosuppression. There was no difference in T-cell proliferation between the individual groups that received plain or DNA-loaded microspheres. Therefore, results for all anionic microspheres formulations or all cationic microspheres formulations at each challenge time point were combined. On a single injection of PLA2/alum, an apparent difference in the SI was seen at week 36 that was not significant ($.13 < P < .84$) when compared with previous time points. Injection of 2 doses of PLA2/alum at week 36 maintained a low T-cell proliferation similar to that observed at weeks 24 and 30 ($P > .5$). In summary, proliferation profiles obtained with mice having received microspheres formulations were similar to those with mice given plasmid DNA alone (data not shown), indicating a low responsiveness of T cells even after extensive boosting.

Activated splenocytes express IL-4, IFN- γ , and the suppressive cytokine IL-10

Secretion of cytokines by T cells determines the T_H1 or T_H2 deviation of the ongoing immune response. This prompted us to analyze the expression of the prototype T_H2 cytokine IL-4, the T_H1 cytokine IFN- γ ,²⁹ and IL-10 considered as a dominant regulatory cytokine^{30,31} by using real-time RT-PCR. Analysis of total RNA from the spleen of mice given PLA2V plasmid DNA showed sustained expression of IFN- γ and IL-10, correlating with data from ELISA performed with the polypeptide recovered in splenocyte culture supernatants.⁸ IFN- γ :IL-4 ratios calculated from $100 \times (1/\Delta Ct)$ values obtained with splenocytes from DNA alone-treated mice were always above 1, indicating a T_H1-biased immune response (Table II). For microsphere formulations, no differences in cytokine expression were found between splenocytes of mice having received plain or DNA-loaded microspheres. Hence, results are presented by combining groups of mice administered either anionic or cationic microspheres. There were no significant differences in cytokine expression between cationic and anionic microspheres (Table II). In contrast with DNA alone, no increase of the IFN- γ :IL-4 ratio was measured by using any microsphere formulation. However, IL-10 expression remained remarkably stable over time for all microsphere formulations.

DISCUSSION

Biodegradable PLGA microspheres have been widely used for vaccination with various antigens, leading to a T_H1-biased immune response with high levels of IFN- γ and low levels of IL-4 and IL-5.⁹ Intradermal gene vaccination of mice induces antigen-specific T_H1 cells that secrete high levels of IFN- γ and stimulate production of antibodies of the IgG2a and IgG3 isotypes, resulting in the reduction of a pre-existing allergen-specific IgE response.⁸ Consistent with this, the combination of DNA plasmid and microspheres contributes to modulate

the immune response in mice, thus leading to prevention of anaphylaxis. The novelty of our data resides in the observation that microspheres alone protect most mice against anaphylaxis through the transient induction of the T_H1 -type antibody IgG2a and on production of IL-10. These markers, as well as antigen-dependent T-cell suppression, are not affected by the presence of DNA, arguing for a dominant immunomodulatory properties of microspheres in the physiological context. Although antibody titers and T-cell proliferation were previously measured in studies examining the potential of antigen-loaded or DNA-loaded microspheres administered to mice, guinea pigs, macaques, and human beings,^{23,24,32,33} the effect of microspheres on subsequent challenges was not assessed. Microsphere-based immunotherapy against allergy was so far limited to the demonstration that (1) IgE titers were reduced after oral delivery of bovine lactoglobulin associated with PLGA microspheres³⁴ and (2) lung eosinophilia was decreased after intranasal or intratracheal administration of plain chitin microspheres.^{35,36}

We show here that there is no synergistic effect of DNA associated with microspheres. Given that the CpG motifs present in the DNA plasmid are expected to contribute to polarize the profile of the immune response, this argues in favor of a strong, and so far not documented, immunodominant effect of the microspheres *in vivo*. This indicates that in the prophylactic setting used, the microspheres are somehow sufficient to instruct the immune system not to overreact at the time the antigen is given during the sensitization procedure. The lack of need for an antigen coding sequence resembles the observation we made when using DNA to vaccinate mice before sensitization with PLA2.⁸ We speculate that microspheres per se could affect the innate immune system and its associated cytokine microenvironment in such a way that it will have a profound effect on the development of acquired immunity. Interestingly, the T_H1 bias observed at the level of the immunoglobulin in the initial period of the treatment is sustained transiently, followed by a mixed T_H1/T_H2 response supported by a similar expression of IL-4 and IFN- γ underlining the plasticity of the modulation *in vivo*. What therefore are the possible mechanisms explaining the protective function of antigen-independent microspheres vaccination? *In vitro*, professional antigen-presenting cells such as DCs engulf cationic microspheres in low acidic phagosomes, resulting in slow degradation that favors expression of the surface marker CD86, and production of TNF- α .^{16,37} In contrast, anionic microspheres found in large phagosomal compartments with normal acidification and degradation rate lead to upregulation of CD83 and CD86 and production of IL-12p40.^{16,37} Together, this indicates a propensity of microspheres to govern maturation of antigen-presenting cells toward a T_H1 type of response, which explains the initial reversal of the T_H2 bias observed in mice. Mice resisting anaphylactic challenges had seric IgE levels slightly below the level reached after sensitization. Moreover, the IgE antibody bound to mast cells implicated in allergic memory³⁸ does not appear to induce anaphy-

lactic reactions either. Given that the increase in IgE titers observed during challenge at week 36 did not trigger anaphylaxis, it is unlikely that the IgE antibody plays a crucial role in this model. Because weak T-cell proliferation was detected in splenocytes recovered immediately after mouse challenge, this argues that suppression seems to be the potent underlying mechanism.

In all groups, we found a sustained production of IL-10 during the whole time frame. IL-10 is a multifunctional cytokine implicated in the limitation and/or termination of inflammatory reactions,^{30,39} explaining why induction of IL-10-producing T cells is a marker of successful specific immunotherapy.⁴⁰ The strong suppressive effects of IL-10 are a result of the inhibition of production of proinflammatory cytokines and downregulation of costimulatory molecules on monocytes,⁴¹ and the reduction of histamine and leukotriene release by mast cells and basophils.⁴² In addition, DCs producing IL-10 or tolerogenic DCs capable of educating T cells to produce IL-10 have been shown to mediate long-lasting tolerance.⁴³

In summary, this is the first report of an antigen-independent and prolonged suppression of an allergic reaction that is modulated by intradermal treatment with microspheres. This occurs through T-cell hyporesponsiveness, prevention of anaphylactic reactions and maintenance of low titers of IgE, but without complete abrogation of T_H2 -mediated responses. This indicates that immune deviation is not the sole mechanism for tolerance induction in this case, as long as sustained expression of IL-10 is guaranteed in splenocytes. It is conceivable that the administration of microspheres might generate in atopic persons sufficient levels of protective IL-10 without affecting the outcome of other essential T_H1/T_H2 -mediated immune responses involved in protection against parasites and at mucosal surfaces.^{44,45}

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