Enhancing Immunogenicity and Reducing Dose of Microparticulated Synthetic Vaccines: Single Intradermal Administration

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Purpose. Our purpose was to evaluate the ability of a polymeric vehicle to release a model synthetic vaccine to the skin in order to reach a potent activation of the specific immune response.

Methods. The peptide-loaded poly-D,L-lactide-co-glycolide acid (PLGA) microparticles were prepared by a double emulsion technique and administered to Balb/c mice. The immune response (antibody and T cell activation) obtained by the intradermal (i.d.) and the subcutaneous (s.c.) routes was tested.

Results. When similar doses of peptide-loaded microparticles were injected s.c. or i.d. in mice, the antipeptide IgG antibody immune response was found to be significantly higher after i.d. injection into the skin. We could also reduce the dose of antigen 10 times by the i.d. route and find a similar antibody response to that obtained by the s.c. immunization. At the lowest i.d. dose level, the IgG2a/IgG1 ratio was also incremented and the IgE production decreased. The i.d. microparticles induced, at both dose levels, a marked IFN-γ secretion by peptide-stimulated splenocytes and lymph node cells and a significant T cell proliferation in spleen cell cultures.

Conclusions. The results demonstrate that peptide-loaded microparticles were efficiently administered by the i.d. route because lower doses were required and powerful antibody and T cell responses were obtained compared to the conventional s.c. administration.

KEY WORDS: immune response; intradermal route; microparticles; synthetic peptide vaccine.

INTRODUCTION

Despite the exciting perspectives of safety, immunological specificity, and low cost and ease of production demonstrated by synthetic peptide vaccines since the early 1980s (1,2), after more than 20 years no marketable vaccines based on this type of antigen have yet been introduced. Major drawbacks of short peptide vaccines derive from their poor stability to proteolytic enzymes *in vivo* and their weak immunogenicity with the conventional aluminum adjuvants. These problems led to the quest of strategies to avoid the *in vivo* hydrolysis of the peptides and to allow their efficient uptake by the immune cells. In essence, two types of approaches to enhance the peptide potency have been applied (3). The first includes chemical changes on the antigenic molecule, such us peptide polymerization, conjugation with carrier lipids or peptides, or changes in the CO–NH peptide bonds; the second is based on the application of new adjuvants, for instance CpG motifs or other bacterial compounds, saponins (QS21) and lipid formulations (MF59, Montanide), or the inclusion or adsorption of the antigen in liposomes or microparticles.

The microencapsulation of synthetic peptides in poly-D,L-lactide-co-glycolide acid (PLGA) polymers, as one of the above-mentioned approaches to enhance peptide stability and immunogenicity, has frequently led to good antibody responses (4,5) as well as cytotoxic T lymphocyte (CTL) activity (6) and protection against challenges (7). The particulation of the antigens, together with the slow biodegradation rate of the formed microparticles, allows their sustained uptake by the antigen-presenting cells (APCs) (8). The controlled release of antigen obtained with polymer microparticles has also been postulated as a mechanism to imitate multiple boosts with a single immunization (4).

In the current research, our strategy consisted in placing the particulate antigen intradermally, close to the more efficient APCs; the specialized population of dendritic cells of the epidermis (called Langerhans cells). These cells cover a great surface area through the skin with their protrusions, capture the antigen *in situ,* and migrate to T-dependent lymphoid organs, wherein they present the antigen and sensitize antigen-specific T cells (reviewed in Ref. 9). As a result, stronger humoral and cellular immune responses than elicited by conventional vaccines have been reported which allow a significant dose reduction for the achievement of similar or superior serum antibodies (10,11). Among the few reports applying synthetic antigens into the skin, most of them used the granulocyte-macrophage colony stimulating factor (GM-CSF) cytokine as adjuvant. For instance, this strategy increased the antigen-specific CTL response against tumors such as melanoma (12) and also improved the antibody production against HIV peptides (13). The superiority of the intradermal (i.d.) route over the subcutaneous (s.c.) one to activate efficiently CTL responses against viral synthetic peptides was also recently shown by means of liposomes and CpG as adjuvants (14).

To our knowledge, this work represents the first study employing PLGA microparticles for the i.d. administration of synthetic peptides. The purpose of this study was to characterize the immune response provided by the single i.d. vaccination as compared to the s.c. immunization with a synthetic peptide vaccine encapsulated in PLGA microparticles.

MATERIALS AND METHODS

Antigen and PLGA Microparticles

The SPf66 antigen (malaria synthetic vaccine) (15), provided by the Fundación Instituto de Inmunología de Colombia (FIDIC, Bogotá, Colombia), was prepared by the multiple solid-phase synthesis method described by Houghten (16).

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ABBREVIATIONS: APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; DMSO, dimethylsulfoxide; i.d., intradermal; MTT, 3-(4,5.dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PLGA, poly-D,L-lactide-co-glycolide acid; s.c., subcutaneous.

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PLGA microparticles were formulated using a water/oil/water solvent extraction technique (7). Briefly, 250 μL of a 100 mg/ml solution of SPf66 in distilled water was emulsified in 5 ml of methylene chloride containing 250 mg of PLGA 50:50 (lactic:glycolic acids) (MW 102,900) or PLGA 75:25 (MW 92,000) (Boehringer Ingelheim, Germany) using a microtip sonicator for 30 s at 60 W (250 Sonifier, Branson, CT, USA). Blank microparticles were prepared by omitting the SPf66 peptide from the aqueous phase of the emulsion. The resulting primary emulsion was added into 25 ml of 8% w/v polyvinyl alcohol (PVA; Mw 30-70,000; Sigma Chemicals, St. Louis, MO, USA) and was further homogenized with a turbine (Ultra-Turrax T 25, IKA-Labortechnik, Staufen, Germany) at 9500 r.p.m. to form the secondary emulsion. This emulsion was poured into 50 ml of 2% isopropanol and stirred for 1 h to extract the organic solvent and complete microparticles hardening. The produced microparticles were then collected by centrifugation (10,000*g* for 10 min at 4°C), washed three times with distilled water to remove residual PVA, resuspended in double-distilled water, and freeze-dried for 24 h.

Particle size, as determined by light diffraction (Coulter LS130 particle size analyzer, Amherst, MA, USA), presented a 95% distribution between 0.8 and 2.0 μm for all the peptide-loaded and blank formulations. Peptide loading was determined using a bicinchoninic acid assay (micro-BCA, Pierce Co., Rockford, IL, USA) after disrupting the microparticles in 0.2 M NaOH. The antigen loadings (means of three batches of microparticles) were measured by this method as 8.4% w/w (PLGA 50:50 formulations) and 7.7% w/w (PLGA 75:25). The encapsulation efficiency, expressed as the percentage of the initial peptide load recovered into microparticles, was close to 80% for both formulations.

Immunization Procedures and Sample Collection

Three groups of 10–11 Balb/c mice (7 weeks old) (Harlan, Barcelona, Spain) were immunized with the microencapsulated SPf66 peptide as detailed in Table I. The first group was subcutaneously injected with a single dose of 100 μg of SPf66 entrapped into microparticles (1:1 mixture of SPf66-loaded formulations made of PLGA 50:50 and PLGA 75:25) suspended in 250 μL PBS. The other two groups of mice received a single dose of 100 or 10 μg of SPf66 in microparticles (same mixture) each by the i.d. route. The vaccine, suspended in 50 μL of PBS buffer, was administered in four injections to the shaved back skin of the animals using a standard disposable 28-gauge insulin syringe. A visible raised cutaneous swelling was regarded as evidence for success of i.d. administration. Two groups were included as controls: 5 mice were injected with microparticles containing no antigen, and 10 mice were injected s.c. with 100 μg of SPf66 adsorbed onto alum adjuvant ($AlOH_3$ gel, FIDIC). These animals were boosted 3 and 6 weeks later with the same alum formulation and SPf66 dose, resembling the schedule previously used for clinical trials (15).

Table I. Immunization Protocols in Balb/c Mice

Group	Route	Schedule (days)	SPf66 (μg/dose)	Formulation
BMP	i.d.	0	0	Blank microparticles
SC/100	s.c.	0	100	Microparticles
ID/100	i.d.	0	100	Microparticles
ID/10	i.d.	0	10	Microparticles
Alum/3 × 100	s.c.	0, 21, 42	100	alum gel

i.d., intradermal; s.c., subcutaneous.-

Blood samples were drawn at 0 (preimmune sera), 3, 6, 9, 12, and 20 weeks, following accepted procedures (17). At 12 weeks, 3 animals of each group were sacrificed after bleeding in order to complete studies on cytokine secretion and cell proliferation. Inguinal, brachial, and axillary lymphatic nodes were removed and pooled, and single-cell suspensions were prepared by passing the homogenized tissues through a Falcon 40 μm nylon cell strainer (Becton Dickinson, Bedford, MA, USA). Spleens were also collected, pooled, and homogenized, and cell suspensions were prepared by similar methods after removing red blood cells with 0.1 M NH_4Cl.

Antibody Determination

The ELISA for the serum antibodies was performed essentially as previously described (7), with SPf66 peptide adsorbed to the ELISA plates (15 μg/mL in PBS buffer) followed by incubation with the sera of the immunized mice. The levels of antibodies were quantified with the following peroxidase-conjugated antibodies: goat anti-mouse IgG (Sigma), goat anti-mouse IgG1 and IgG2a (Southern Biotechnology Inc., Birmingham, AL, USA), and goat anti-mouse IgE (Nordic Immunological Laboratories, Tilburg, The Netherlands). After incubation with ABTS substrate buffer (Sigma), the optical density values were measured at 405 nm on an automatic microplate reader (Multiscan EX, Labsystems, Helsinki, Finland). The mean and standard deviation (SD) of 6 preimmune sera were calculated on each plate, and antibody-positive cut-off values were set as their mean + 2-fold SD. Anti-SPf66 titers were expressed as the $\log_{10}$ of the reciprocal of the highest dilution giving the positive reaction (over the cut-off value).

Cytokine Determination

Isolated splenocytes or lymph node cells were placed in 24- or 96-well plates, respectively, at a final concentration of 1.5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Gibco BRL, Invitrogen, Paisley, Scotland, UK). All cultures were kept in triplicate, either in presence of the unspecific mitogen concanavalin A (ConA, 2 μg/ml) (Sigma) or the SPf66 synthetic antigen (15 μg/ml). Another triplicate culture was placed in absence of stimuli (only with supplemented medium). Supernatants were collected at 48 h of incubation at 37°C in 5% CO_2 atmosphere and stored frozen at –80°C until assayed.

Murine IFN-γ and IL-4 levels were measured by capture ELISA using OptEIA IFN-γ or OptEIA IL-4 ELISA sets (Pharmingen, Becton Dickinson). Flat-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl mAb specific for either murine IFN-γ or IL-4, diluted in 0.1 M carbonate buffer, pH 9.5. Plates were washed with PBS/Tween 20 (PBST) and blocked with 200 μl of PBS supplemented with 10% fetal bovine serum (PBS-FBS) for 1 h. Following wash with PBST, 100 μl of sample

supernatants were placed into the appropriate wells and incubated 2 h at room temperature. Plates were washed with PBST, and 100 μl of biotinylated rat anti-mouse cytokine (IFN-γ or IL-4) mAb, conjugated to avidin-HRP, were added and incubated for 1 h at room temperature. After the final wash with PBST, to reveal the reaction, 100 μl of TMB substrate solution (Pharmingen, Secton Dickinson, San Diego, CA) were incubated for 30 min. To stop the reaction, 50 μl of 2 N H_2SO_4 were added, and the absorbance was read at 450 nm. Cytokine levels were determined by comparison with standard curves generated from murine recombinant cytokines (IFN-γ and IL-4) after log/log linear regression analysis.

Colorimetric Assay for Cell Proliferation

Cell proliferation assays were performed on splenocyte cultures using the MTT colorimetric method, based on the reduction of the MTT salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) to colored formazan (18). This simple assay has been shown as reliable and reproducible for lymphocyte proliferation studies (19) and avoids the use of radioactive reagents. Briefly, spleen cells were seeded 1.5×10^5 cells per well in 100 μl of supplemented RPMI 1640 culture medium in 96-well microtiter plates. SPf66 peptide was added to 5 wells at a final concentration of 15 μg/ml, concanavalin A was added to other 5 wells at a final concentration of 2 μg/ml, and 5 more wells were cultured without any stimulus. The plates were incubated at 37°C in 5% CO_2 atmosphere for 36 h. After addition of 30 μl of 5 mg/ml MTT (in PBS buffer) per well, cultures were incubated for 4 h for the dye to be metabolized. The microtiter plates were centrifuged (500 × *g*, 4°C, 10 min), supernatants removed carefully with pipette, and 100 μl of DMSO were added to each well. The optical density of the product was evaluated 15 min later using a microplate reader at 560-nm wavelength against the reference 690-nm filter.

RESULTS

Specific Antibody Response in Serum

Specific antibody response and antibody production profile in each group of mice were evaluated over a period of 20 weeks. As shown in Fig. 1, single i.d. immunization with 100 μg of SPf66 entrapped in microparticles generated high and sustained titers of systemic anti-SPf66 IgG antibody, significantly greater than those observed following a single s.c. injection of the same dose of microparticulated antigen. The low dose of 10 μg of SPf66 by the i.d. route induced similar IgG levels compared to the former s.c. group. The three microparticle-immunized groups presented significantly higher IgG titers compared to those observed following the conventional triple s.c. immunization with alum, from 3 up to 12 weeks of the study ($p < 0.01$, Mann–Whitney *U* test).

Antibody isotype data, represented in Table II, imply that the use of microparticles induced detectable IgG2a levels in all mice. As previously shown, the subcutaneous immunization with the SPf66 antigen adsorbed to alum adjuvant only elicited IgG1 isotype (20). Both i.d. and s.c. immunizations with microparticles produced similar levels of IgG2a, independently of the dose level. The IgG2a to IgG1 ratio of 0.9 obtained in mice immunized intradermally with the lower

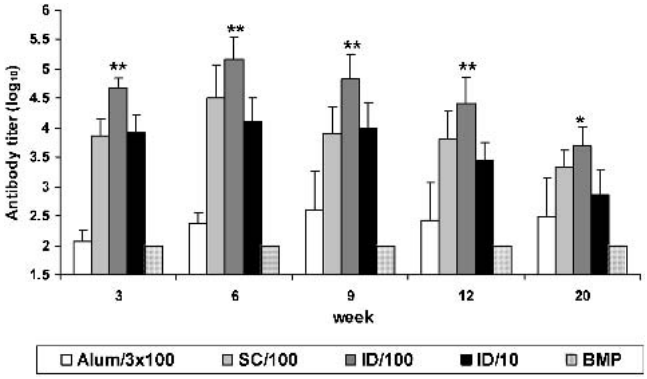

Fig. 1. Anti-SPf66 IgG antibody in sera of Balb/c mice after single administration of microparticle-based vaccine or triple injection of alum-based control vaccine (see Table I for further details on groups of mice). Data are presented as the mean ± SD (error bars) of the titer values of 10–11 mice (weeks 3–12) or 7–8 mice (week 20). * $p < 0.05$, significant against SC/100 group. ** $p < 0.01$, significant against SC/100 group (Mann–Whitney *U* test).

dose (10 μg) of microencapsulated SPf66 was significantly higher ($p < 0.01$, Mann–Whitney *U* test) than the ratios elicited with the higher dose level (100 μg) by both i.d. and s.c. routes (Table II). These results demonstrate that the IgG isotype profile reached by immunization with microparticles is affected by the dose of entrapped antigen.

As shown in Table II, anti-SPf66 specific IgE titers were detected in sera of the immunized mice. IgE levels were minor for mice receiving subcutaneous SPf66-alum and also for mice i.d. immunized with the low dose of microparticulated vaccine. Animals that received 100 μg of SPf66 in microparticles secreted consistent levels of IgE, significantly greater ($p < 0.01$) than those obtained for the first two groups. IgE titers presented a wide variability in sera of s.c. immunized mice.

Cytokine Secretion

As shown in Fig. 2, the i.d. immunization with a single administration of 100 or 10 μg of SPf66-loaded microparticles induced spleen and lymph node T cells that secreted high levels of IFN-γ in response to stimulation with soluble SPf66 *in vitro*. The activation of T cells to secrete IFN-γ seemed to be higher for the lymph node T cells than for splenocytes. On the other hand, the levels of IFN-γ were low in both cultured cell types following s.c. immunization with microparticles or alum, as also was the secretion of the control animals that received blank microparticles. The nonstimulated cultures (negative controls) secreted undetectable IFN-γ amounts. All SPf66-stimulated cultures secreted undetectable levels of IL-4 (<7.8 pg/ml).

Splenocyte Proliferation

Proliferative responses were detected in pooled spleen cells from the two groups immunized by the i.d. route with SPf66-loaded microparticles. Three immunizations of 100 μg of SPf66 in alum or single immunization with SPf66 microparticles by the s.c. route produced no significant proliferative responses, as shown by the MTT assay (Fig. 3). As a control,

Table II. Isotypes of Anti-SPf66 Antibodies (log_{10}) at 12 Weeks Postimmunization (Mean ± SD)

Group*	Route	SPf66 (μg/dose)	Anti-SPf66 antibody			IgG2a/IgG1†	IgE/IgG1†
			IgG1	IgG2a	IgE		
BMP	i.d.	0	<2 (0/5)	<2 (0/5)	<2 (0/5)	—	—
SC/100	s.c.	100	4.3 ± 0.5 (10/10)	3.1 ± 0.5 (10/10)	2.8 ± 0.9 (6/10)	0.74 ± 0.13	0.65 ± 0.18
ID/100	i.d.	100	5.0 ± 0.4‡ (11/11)	3.4 ± 0.6 (11/11)	3.5 ± 0.4 (11/11)	0.68 ± 0.11	0.70 ± 0.12
ID/10	i.d.	10	3.7 ± 0.5 (10/10)	3.4 ± 0.4 (10/10)	2.5 ± 0.6 (5/10)	0.94 ± 0.17‡	0.67 ± 0.13
Alum/3 × 100	s.c.	100	3.4 ± 0.9 (9/10)	<2 (0/10)	2.1 ± 0.6 (2/10)	—	0.61 ± 0.16

i.d., intradermal; s.c., subcutaneous.
* Immunization protocols defined in Table I.
† Mean ± SD ratio of the individual ratios calculated for each mouse of the group.
‡ $p < 0.01$, significantly greater as compared to the rest of the values of the column (Mann–Whitney *U* test).

the administration of blank microparticles produced an undetectable proliferative response.

DISCUSSION

A consistent improvement on the immunogenicity of synthetic peptides would probably show the way to the market to these immunologically defined, safe, and economical vaccines. Once we established the efficient adjuvant effect achieved through the encapsulation of a model synthetic antigen into PLGA microparticles by s.c. or mucosal administration (7,20), we then studied the effect of the specific targeting of these polymer formulations to the skin. We chose the dermal tissue to target the particulate vaccine because it has been well-characterized as a site to elicit potent immune responses (21). The administration of microparticles by the i.d. route was here associated with higher antibody responses, superior to those obtained with microparticles by the s.c. route. Moreover, the i.d. route was particularly efficient as even the lowest antigen dose (10 μg) induced antibody responses comparable to 100 μg by the s.c. route (Fig. 1). From these results it seems clear that the i.d. vaccination with synthetic peptide-loaded PLGA microparticles can successfully achieve high and sustained antibody titers in a single administration. Puri *et al.* previously showed that muramyl dipeptide (MDP)-loaded ovalbumin (OVA) microparticles administered in 90–100 injections using an intradermal delivery device were more effective than the s.c. administration in inducing anti-OVA IgG antibody titers (22). These authors, using a model of fluorescent latex microparticles, associated their results to an efficient lymph node targeting by the i.d. route, and they also confirmed the previously reported *in vitro* observation that polymer-conjugated antigen delivery to dendritic cells results in a more efficient antigen presentation compared to the administration of the soluble antigen (8). Besides, one of the few experiments in which PLGA microparticles were i.d. administered evidenced their uptake by dendritic cells *in vivo* without the use of immunomodulators (23).

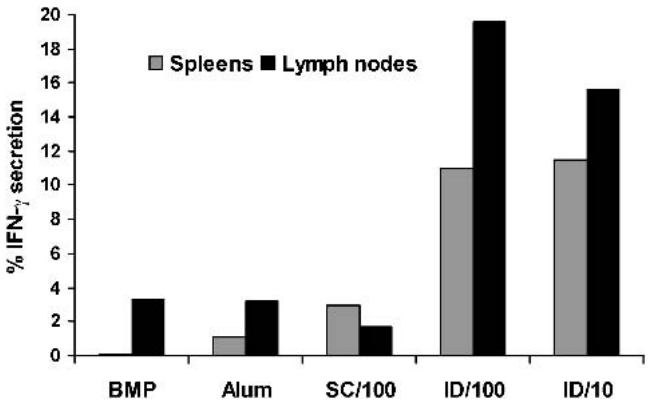

Fig. 2. IFN-γ secretions in supernatants of splenocytes or lymph node cells of immunized mice (groups of Table I) at 48 h poststimulation. To normalize the values between groups, results are expressed as the percentage of IFN-γ production in triplicate wells stimulated with SPf66 relative to the production of the wells stimulated with ConA.

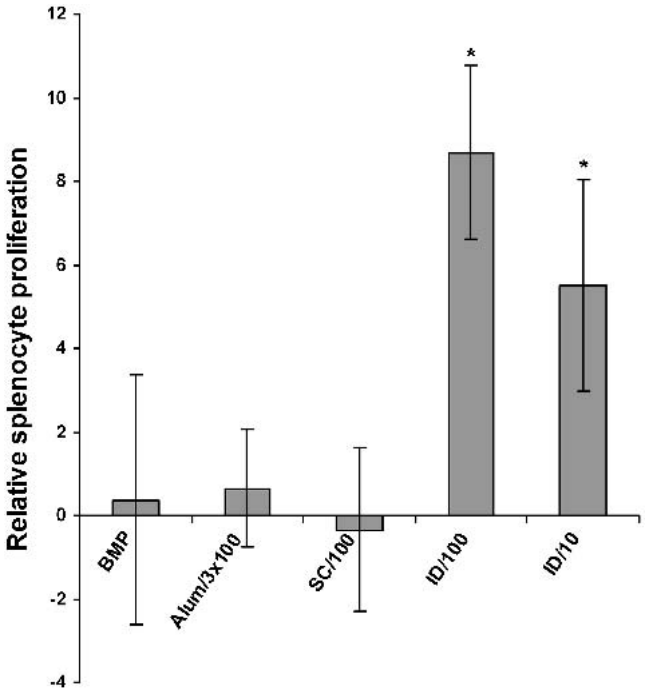

Fig. 3. Proliferation of stimulated splenocytes (groups of Table I) calculated by the MTT assay. Results were standardized by dividing the absorbance values obtained for SPf66-stimulated cultures (increments with reference to the absorbances of nonstimulated cultures) by those of the ConA-stimulated controls as the maximum value. Represented values are the percentage of absorbance for SPf66-stimulated cultures relative to the absorbance of ConA-stimulated ones. Mean values (±SD) of five replicates are shown. * $p < 0.05$, significant against BMP, Alum/3x100 and SC/100 groups (Mann–Whitney *U* test).

Given that vaccination with microparticles induced a strong B-cell response in our study, the next step was to characterize the variety of antibodies and cytokines involved in the response to determine the T helper (Th) subset activated by the vaccination protocols. Differences in antibody isotype are associated with different types of Th cells. In general, Th1 immune responses promote the production of IgG2a antibody in mice whereas Th2 immune responses promote the production of IgG1 antibody. Th cells are also characterized by the lymphokines they produce, with the signature cytokine for Th1 being IFN-γ and that for Th2 cells being IL-4. The Th1 subset is associated to cell-mediated immune responses while the Th2 subset induces humoral-type immune responses (24). We have already shown in previous studies on the SPf66 antigen that alum and PLGA adjuvants generate distinct antibody responses, with alum producing solely the IgG1 subclass and microparticles eliciting a significant secretion of the IgG2a antibody (20). In this study, the ratio IgG2a/IgG1 is similar for both i.d. and s.c. administration of the higher dose of microparticles (Table II). The ratio of the lowest dose (i.d.) was found higher, in concordance with the rule that, for antigens able to induce Th1 and Th2 responses, lower doses favor the Th1 component (24). The IgE component of the antibody response presented in our results appears significantly higher after the i.d. administration of the 100-μg dose as compared to the rest of the groups. The switching to the IgE antibody is attributed to the Th2 subset activation and has been correlated to the cutaneous repeated exposure to protein antigens (25). Remarkably, the IgE secretion was well-correlated with IgG1 isotype in each group, as shown by the similarity of the IgE/IgG1 ratios (Table II). Mouse IgG1 competes with IgE for binding to the same antigen epitopes, and this way, IgG1 is able to inhibit IgE binding and to suppress a hypothetic allergic reaction (26).

As determined in splenocyte and lymph node cell stimulated cultures and shown in Fig. 2, the observation that the i.d. administered particles produced relatively high IFN-γ secretion compared to the low secretion elicited by s.c. microparticles reflects a Th1-type response activation. The highest IFN-γ production in response to the SPf66 antigen was determined for lymph node cells of i.d. immunized mice at both dose levels. This could suggest that an efficient dendritic cell targeting is achieved by the immunization into the skin, and their migration to the draining lymph nodes results in a very efficient activation of the Th1 cell subset. The undetectable levels of IL-4 also reflect a Th1-type cytokine profile for i.d. groups. The finding of evident splenocyte proliferation promoted by the i.d. administered microparticles (Fig. 3), in contrast to the lack of proliferation by the s.c. route, shows that the delivery of microparticles to the skin leads to the activation of T cells in the systemic immune organs. This suggests that the IFN-γ cytokine production induced high Th1 cell proliferation. The similar levels of cytokine production and lymphoproliferation reached with the lowest dose of i.d. microparticles compared to the highest one confirms the high efficiency of the i.d. route to reduce dose without losing efficiency (10,11).

The use of needles and the requirement of expert technicians to administer the vaccine would be the main weakness of the approach we propose here, as ideal vaccines should be administered easily, by noninvasive tools. As for the solution to this problem, novel delivery systems are currently under investigation to administer antigens to the skin, using needle-free devices as jet injectors (27), micromechanical disruptors based on silicon projections (28), or patches with absorbed toxins as adjuvants (29). Nevertheless, our results show for the first time that biodegradable microparticles have potential to target efficiently synthetic peptides to the dendritic cells of the skin; the only parenteral site that can itself act as an immune organ. The immune response generated by this strategy may be suitable for the generation of synthetic vaccines against pathogens in which both antibody and Th1 cells work as a protective mechanism.

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