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Comparison of Plasmid Vaccine Immunization Schedules Using Intradermal *In Vivo* Electroporation[▽]

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In vivo electroporation (EP) has proven to significantly increase plasmid transfection efficiency and to augment immune responses after immunization with plasmids. In this study, we attempted to establish an immunization protocol using intradermal (i.d.) EP. BALB/c mice were immunized with a plasmid encoding HIV-1 p37Gag, either i.d. with the Derma Vax EP device, intramuscularly (i.m.) without EP, or with combinations of both. A novel FluoroSpot assay was used to evaluate the vaccine-specific cellular immune responses. The study showed that i.d. EP immunizations induced stronger immune responses than i.m. immunizations using a larger amount of DNA and that repeated i.d. EP immunizations induced stronger immune responses than i.m. priming followed by i.d. EP boosting. Two and three i.d. EP immunizations induced immune responses of similar magnitude, and a short interval between immunizations was superior to a longer interval in terms of the magnitude of cellular immune responses. The FluoroSpot assay allowed for the quantification of vaccine-specific cells secreting either gamma interferon (IFN-γ), interleukin-2 (IL-2), or both, and the sensitivity of the assay was confirmed with IFN-γ and IL-2 enzyme-linked immunosorbent spot (ELISpot) assays. The data obtained in this study can aid in the design of vaccine protocols using i.d. EP, and the results emphasize the advantages of the FluoroSpot assay over traditional ELISpot assay and intracellular staining for the detection and quantification of bifunctional vaccine-specific immune responses.

Vaccination with genes was first described in the early 1990s and is becoming an alternative to traditional vaccine strategies. DNA vaccines possess several advantages, such as the capacity to induce a balanced immune response including humoral as well as cellular immune responses similar to those induced during natural infection with intracellular pathogens. The potential of DNA vaccines has been shown in numerous preclinical studies and by the licensure of veterinary DNA vaccines against infectious diseases and cancer (3, 9, 20). However, immunogenicity has been limited in humans, and ways to enhance the potency of these vaccines are being investigated. Besides gene optimization and the use of adjuvants (17), the most promising approach for plasmid vaccines administered as a single modality is by the use of *in vivo* electroporation (EP). EP has been shown to considerably increase the transfection efficacy of plasmid vaccines, ultimately leading to enhanced and long-lasting expression (10, 24) and improved immunogenicity (13, 21, 27, 28) of the encoded antigen. Furthermore, the electric pulses cause mild inflammation, with resulting recruitment of antigen-presenting cells (APCs) to the site of injection (19, 24), which further enhances the immunogenicity.

Although intramuscular (i.m.) delivery of DNA vaccines, with or without the addition of EP, has been studied most extensively, DNA vaccine delivery to skin is becoming increasingly popular. Unlike muscle tissue, the dermal tissue has a large population of resident APCs, including Langerhans cells and dermal dendritic cells, that can facilitate the induction of vaccine-specific immune responses (2, 16). There is also a more rapid turnover of cells in the skin than in muscle, which together with the large number of APCs can lead to a rather fast removal of plasmids from the site of injection (24). This feature is positive for vaccination, as transient expression of the encoded antigen is sufficient to induce strong immune responses. The rapid removal of vaccine plasmids might also explain why more DNA is usually required to induce the same level of expression as that induced by i.m. delivery (10, 15). The skin is also an assessable tissue, making both monitoring and evaluation of immune responses easy to perform. More importantly, the addition of EP after intradermal (i.d.) delivery appears safe, as it does not affect the persistence or integration of vaccine plasmids (5, 24).

Laddy et al. conducted a head-to-head comparison of EP-augmented i.m. and i.d. delivery of equal amounts of influenza virus-encoding vaccine plasmids in rhesus macaques. Immune reactivity assessed after three immunizations revealed that i.m. EP induced the highest levels of cellular immune responses, whereas i.d. EP was superior for induction of cross-reactive and neutralizing antibodies (18). This observation was confirmed by a more recent publication (12), and the results clearly reflect the difference in immunological properties between muscle and dermal tissue also when applying EP.

A number of clinical trials using EP-augmented DNA vaccine delivery have been or are being conducted (21, 28; http://clinicaltrials.gov/ct2/results?term=electroporation), and even

though the majority of studies have employed i.m. EP, a few trials have used i.d. EP. Regardless of which route is used, the immunization protocols differ in terms of the number of immunizations and the interval between them. Hence, in this study, we investigated various parameters for i.d. immunization of mice by using a plasmid encoding HIV-1 Gag as a model immunogen which induces both cellular and humoral responses in mice (7) as well as in humans (25; B. Wahern et al., unpublished data). The different parameters of immunization being assessed were i.d. EP compared to i.m. injection without EP, the choice of priming (i.m. or i.d. EP), the number of immunizations (one, two, or three), and the interval between immunizations (4 or 8 weeks).

Based on these parameters, a number of immunization protocols were assessed for the induction of antigen-specific antibodies and cellular vaccine-specific responses as measured by secretion of gamma interferon (IFN-γ) and/or interleukin-2 (IL-2), using a novel FluoroSpot assay. Results from the FluoroSpot assay were compared to those of conventional IFN-γ and IL-2 enzyme-linked immunosorbent spot (ELISpot) assays. The FluoroSpot assay quantifies cytokine secretion in a sandwich immunoassay similar to ELISpot assay but uses fluorochrome-labeled detection reagents which enable simultaneous detection of two cytokines in the same well. Analysis in an automated FluoroSpot reader provides information about the number of cells secreting IFN-γ and IL-2 as well as the number of bifunctional T cells cosecreting both cytokines, which is important because the number and combinations of cytokines being produced by a single cell can indicate the quality of the T cell response of that cell (1, 4).

The study showed that a straightforward protocol using repeated i.d. EP immunizations with a short immunization interval induced strong and long-lasting immune responses. Furthermore, we demonstrated that the FluoroSpot assay is as sensitive as the conventional ELISpot assay and can thus serve as a potent alternative for assessing bifunctional cellular immune responses.

MATERIALS AND METHODS

Groups of 8 to 10 female BALB/c mice, 5 to 9 weeks old, were immunized at weeks 0, 4, and 8 with 15 µg of a plasmid encoding HIV-1 p37GagB (26) i.d. with the Derma Vax EP device (CytoPulse Sciences/Cellectis, Romainville, France) (on one flank of the mice, as described in reference 24) or with 50 µg of the same plasmid i.m. without EP. Ten days after the last immunization, all mice were sacrificed and spleens and sera were collected. The experiment was repeated for mice receiving one, two, or three i.d. EP immunizations with 4-week immunization intervals. These mice were bled regularly from the tail vein to obtain sera and peripheral blood mononuclear cells (PBMCs). PBMCs and splenocytes were purified by Ficoll-Paque separation (GE Healthcare AB, Stockholm, Sweden), and cellular immune responses were measured using IFN-γ and IL-2 ELISpot and FluoroSpot assays according to the manufacturer's guidelines (Mabtech AB, Nacka Strand, Sweden).

Briefly, the polyvinylidene difluoride (PVDF) plates used for both assays were treated with ethanol prior to antibody coating. In ELISpot assays, plates were coated with either monoclonal antibody (MAb) AN18 for IFN-γ detection or MAb 1A12 for IL-2 detection (1.5 µg/well). In FluoroSpot assays, low-fluorescence plates were coated simultaneously with MAbs AN18 and 1A12 (1.5 µg/well/MAb). A total of 10^5 cells were plated per well and then stimulated for 20 h with either a pool of overlapping peptides covering HIV-1 p24GagB (15-mers with 10-amino-acid overlaps), H-2K^d-restricted peptide AMQMLKETI (present in p24GagB), or H-2D^d-restricted peptide VGPTPVNII (present in HIV-1 protease; control peptide). The final concentration of peptides was 5 µg/ml/peptide. In FluoroSpot assays, a costimulatory anti-CD28 MAb (0.1 µg/ml) was added to the cells during incubation. ELISpot plates were developed with biotinylated

TABLE 1. Immunization protocols

Group	Immunization[a] at week:		
	0	4	8
1× EP			EP
2× EP (4 wk)		EP	EP
3× EP	EP	EP	EP
2× EP (8 wk)	EP		EP
2× IM + 1× EP	i.m.	i.m.	EP
1× IM + 1× EP	i.m.		EP
3× IM	i.m.	i.m.	i.m.
1× IM			i.m.
Naïve			

[a] EP, i.d. EP immunization (15 µg DNA); i.m., immunization without EP (50 µg DNA).

detection MAb R4-6A2 (1 µg/ml) or MAb 5H4 (1 µg/ml) followed by streptavidin-alkaline phosphatase (ALP) and 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP/NBT) substrate. In FluoroSpot assays, bound cytokines were detected using fluorescein isothiocyanate (FITC)-labeled MAb R4-6A2 and biotinylated MAb 5H4 followed by anti-FITC antibody conjugated to a green fluorochrome and streptavidin conjugated to a red fluorochrome. The numbers of spot-forming cells (SFCs) in the ELISpot and FluoroSpot assays were determined by using an iSpot reader (AID GmbH, Strassberg, Germany) with software enabling overlay analysis of cells secreting both cytokines.

As a readout of humoral responses, we performed an anti-Gag enzyme-linked immunosorbent assay (ELISA) as previously described (11). Plates were coated with 100 µl/well of 1-µg/ml recombinant HIV-1 p24Gag (FIT Biotech, Tampere, Finland) or HIV-1 gp160B (MicroGeneSys Inc., CT) as a control antigen. Statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software, CA), and a two-tailed Mann-Whitney test was used to analyze differences between groups.

RESULTS AND DISCUSSION

Mice were immunized with a plasmid encoding HIV-1 p37Gag according to the protocols shown in Table 1. In addition to evaluating the IFN-γ/IL-2 FluoroSpot assay (Fig. 1), the different parameters explored were i.d. EP compared to i.m. injection without EP, the choice of priming (i.m. or i.d. EP), the number of immunizations (one, two, or three), and the interval between immunizations (4 or 8 weeks). The choice of comparing i.m. immunization with i.d. EP immunization and using i.m. immunization for priming immunizations was made because i.m. immunization with a needle has been the gold standard for DNA vaccine delivery.

Although a larger amount of DNA was used for the i.m. immunizations (50 µg), i.d. EP immunizations (15 µg) induced stronger ($P < 0.01$) IFN-γ, IL-2, and IFN-γ-plus-IL-2 responses (Fig. 2). The difference between the two modes of immunization was true for both one and three immunizations. In terms of antibody responses, three but not one immunization by i.d. EP induced higher ($P < 0.01$) antibody titers than the same number of i.m. injections (Fig. 3). Furthermore, one i.d. EP immunization induced similar cellular and antibody responses to those induced by three i.m. immunizations (Fig. 2 and 3). These results confirm a large body of studies demonstrating that EP has the potential to decrease the amount of DNA and the number of immunizations needed to induce strong immune responses.

Repeated i.d. EP immunizations were superior to i.m. priming followed by an i.d. EP boost in inducing antibody responses ($P < 0.05$). For cellular responses, however, there was only a
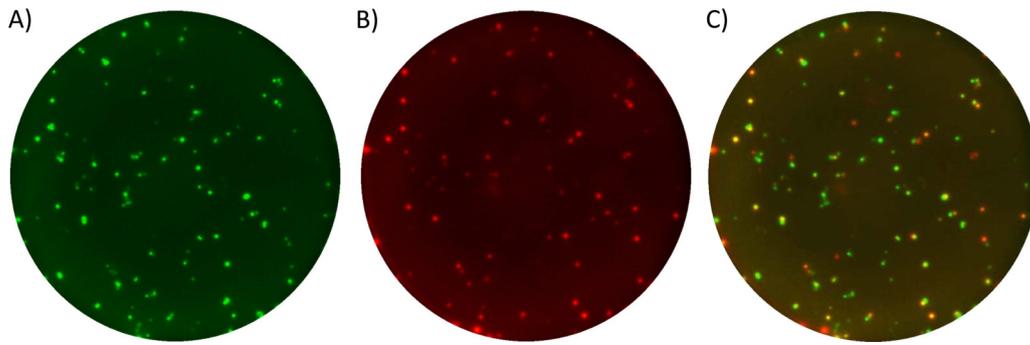
FIG. 1. The IFN-γ/IL-2 FluoroSpot assay detects cells secreting IFN-γ (green) (A), IL-2 (red) (B), and both cytokines (yellow) (C). Panel C is a computerized overlay of panels A and B. The images show responses by splenocytes from a mouse immunized with plasmid-encoded p37GagB and stimulated with a p24GagB peptide pool.

trend in favor of repeated i.d. EP immunizations over i.m. priming and i.d. EP boosting. Buchan et al. conducted a similar comparison where repeated i.m. EP immunizations were compared to i.m. priming and i.m. EP boosting, and they showed that the heterologous prime-boost approach induced superior immune responses to the encoded tumor antigens (8). However, these studies differed in both number of priming immunizations (one versus three) and route of immunization, and perhaps the enhanced antibody responses observed after repeated i.d. EP immunizations in our experiment were a consequence of using the dermal route of immunization, which typically generates superior humoral immune responses (12, 18).

In terms of the number of immunizations, two and three i.d. EP immunizations induced similarly high immune responses, whereas one immunization induced weaker IFN-γ, IL-2, IFN-γ-plus-IL-2 ($P < 0.05$), and antibody ($P < 0.01$) responses than both two and three i.d. EP immunizations. Applying a 4- or 8-week immunization interval did not affect the antibody response. However, an interval of 4 weeks between immunizations was superior to one of 8 weeks in terms of IFN-γ and IL-2 production. This finding contrasts with what was observed when evaluating the timing of DNA boosting immunizations in rhesus monkeys, where vaccine plasmids were administered i.d. or i.m. without EP. In that study, the length of the interval between i.d. priming and boosting immunizations did not significantly affect the antibody responses. In contrast, for i.m. immunizations, antibody responses were significantly stronger with a longer immunization interval (22). Again, the discrepancy in results between studies might be a consequence of the choice of route and method of immunization.

In both FluoroSpot and ELISpot assays, stimulation of cells using the H-2K^d-restricted AMQMLKETI peptide resulted in similar magnitudes of IFN-γ, IL-2, and IFN-γ-plus-IL-2 responses to those obtained using the p24GagB peptide pool, suggesting that the Gag antigen induces mainly CD8$^+$ T cell responses in these settings (data not shown). Neither IFN-γ/IL-2 responses to the HIV PR epitope (VGPTPVNII) nor antibody responses to the HIV gp160 protein were detected in any of the groups (data not shown), confirming that the detected immune responses were antigen specific.

To further distinguish between the immunization schedules inducing the highest immune responses, i.e., repeated i.d. EP

immunizations with a 4-week immunization interval, and to assess the quality of the response, the kinetics of the immune responses were studied. As seen in the previous experiment, two and three immunizations induced similar cellular responses 10 days after the last immunization (Fig. 4). However, for one and three i.d. EP immunizations, the levels of IFN-γ-, IL-2-, and IFN-γ-plus-IL-2-producing cells increased up to day 17, when three immunizations were superior to two. The difference remained for 100 days after the last immunization. IL-2 secretion by splenocytes from mice immunized with two and three i.d. EP immunizations reached the highest levels at day 108. In terms of antibody responses, there was no significant difference between two and three immunizations. However, the difference between single and multiple i.d. EP immunizations was significant ($P < 0.05$) (Fig. 5). Since the three groups included in this experiment were immunized i.d. by EP, the difference in immune response between them was most likely the result of an increased presence of the immunogen after an increased number of immunizations.

This study shows that repeated i.d. EP immunizations as a single vaccine modality can induce strong immune responses. The use of this approach instead of heterologous prime-boost immunizations with microbial vectors or recombinant proteins for boosting immunizations would be beneficial due to fewer regulatory and safety concerns, as well as the ease of development and manufacturing. We previously reported, for mice, that boosting i.d. DNA priming immunizations with EP-augmented DNA immunization generates humoral and cellular superior humoral and cellular immune responses to carcinoembryonic antigen (CEA) compared to boosting with recombinant CEA (6). Plasmid-based vaccines delivered by EP in preclinical experiments have also proved to be superior to viral vectors in some settings. This was demonstrated in a study where rhesus macaques were immunized with either DNA or adenovirus serotype 5 (Ad5) encoding similar simian immunodeficiency virus (SIV) antigens. It was observed that the DNA approach induced higher magnitudes and a more polyfunctional profile of the cellular immune response than the Ad5 approach (14). Additionally, Ad5, as opposed to DNA, was unable to boost the initial immunization, which highlights the issue of antivector immunity after repeated immunizations with viral vectors. This concern is commonly circumvented by DNA priming prior to viral vector boosting (29).
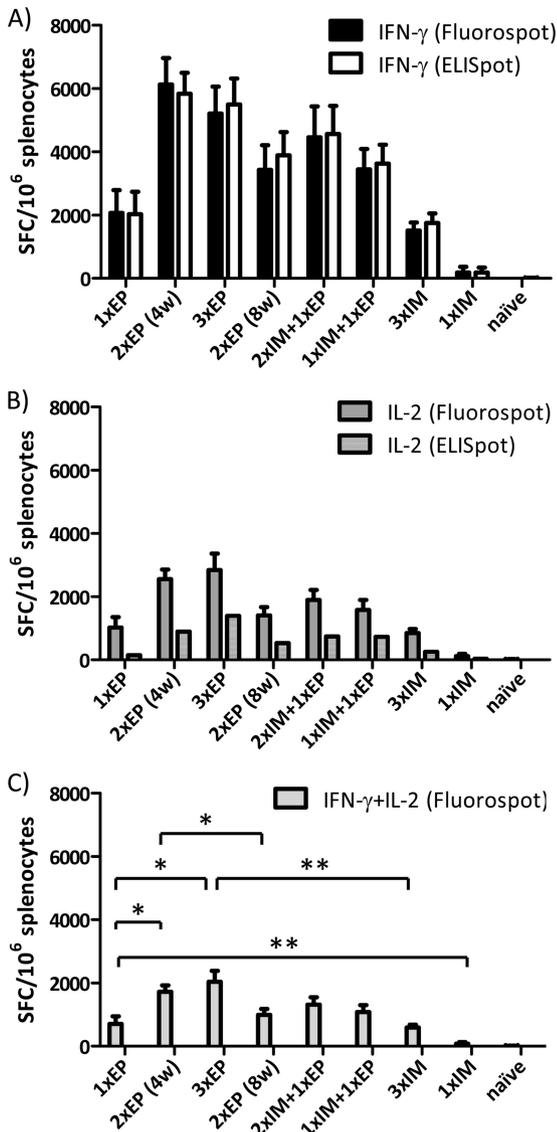
FIG. 2. Cellular responses measured by IFN-γ/IL-2 FluoroSpot assay and IFN-γ and IL-2 ELISpot assays. Individual (FluoroSpot and IFN-γ ELISpot assays) and pooled (IL-2 ELISpot assay) splenocytes were stimulated with a p24GagB peptide pool 10 days after the last immunization. Important significant differences in IFN-γ/IL-2 FluoroSpot results (C) are marked with asterisks (∗, $P < 0.05$; ∗∗, $P < 0.01$), and these also apply to the IFN-γ (A) and IL-2 (B) FluoroSpot assays. Bars and error bars represent means and standard errors of the means, respectively ($n = 8$).

The FluoroSpot and ELISpot assays gave similar results for IFN-γ responses, with no significant difference for any of the nine groups included in this study, confirming the sensitivity of the FluoroSpot assay (Fig. 2A). The higher levels of IL-2 secretion detected in the FluoroSpot assay were most probably due to the costimulatory anti-CD28 antibody that was added to the cells during incubation in order to compensate for the capturing of IL-2 by the anti-IL-2 coating antibodies (Fig. 2B) (23). Hence, the FluoroSpot assay is equal to ELISpot assay in terms of sensitivity and standardization but also possesses the advantages of intracellular staining, generating information re-
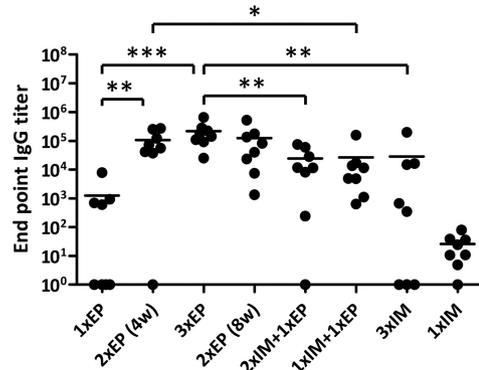

FIG. 3. Antibody responses measured by p24GagB ELISA with sera from individual mice ($n = 8$) collected 10 days after the last immunization. Important significant differences are marked: ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

garding the bifunctionality of the vaccine-induced immune responses.

Taken together, the results of this study show that a straight-forward protocol using repeated i.d. EP immunizations with a
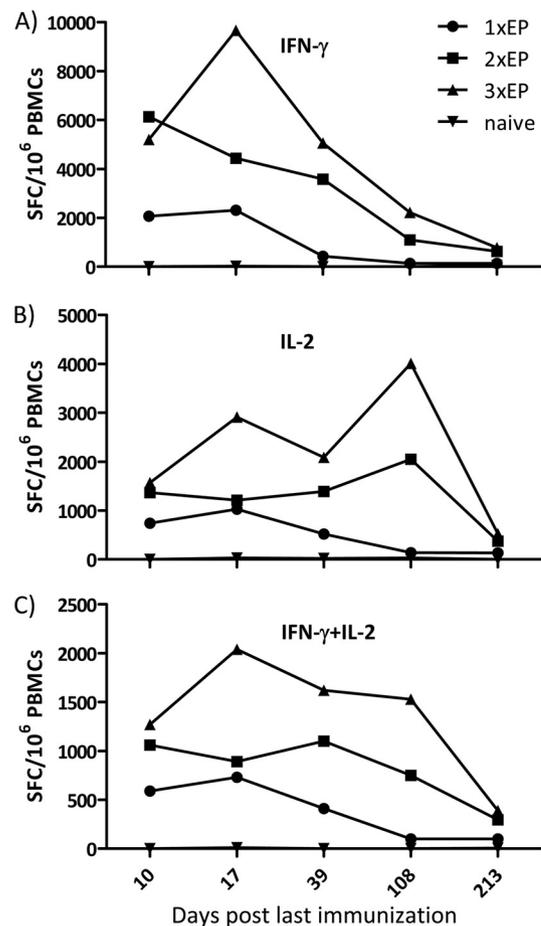

FIG. 4. Kinetics of IFN-γ (A), IL-2 (B), and IFN-γ-plus-IL-2 (C) responses as measured by IFN-γ/IL-2 FluoroSpot assay of pooled PBMCs ($n = 10$) at different time points after the last immunization. Cells were stimulated with a p24GagB peptide pool.
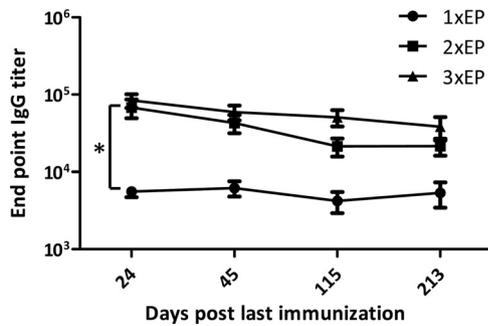
FIG. 5. Antibody titers at different time points after the last immunization, measured by p24Gag ELISA with sera from individual mice. ∗, $P < 0.05$. Error bars represent standard errors of the means ($n = 10$).

rather short immunization interval induces strong and long-lived immune responses, and we believe that these data can facilitate the design of immunization protocols using EP-augmented i.d. DNA immunization. Moreover, we demonstrated that the FluoroSpot assay is as sensitive as conventional ELISpot assay and can thus serve as a potent alternative for assessing bifunctional cellular immune responses.

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