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Enhancement of the effectiveness of electroporation-augmented cutaneous DNA vaccination by a particulate adjuvant

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Abstract

DNA vaccines are attracting increased attention due to multiple advantages over conventional vaccines. Attempts to improve these vaccines focus on enhancing DNA delivery and employing novel immunoadjuvants. Electroporation (EP) has emerged as an effective method for delivering DNA vaccines, significantly enhancing humoral and cellular responses. To further improve EP-augmented DNA vaccination, we used micron-size gold particles as a particulate adjuvant. DNA is not bound, or adsorbed, to the particles. Gold particles were coinjected intradermally with plasmid DNA encoding the hepatitis B virus surface antigen (HBsAg) into mice, both in the absence and presence of noninvasive EP. The particles enhanced the percentage of responding animals, and shortened the time for reaching maximal antibody titers by 2 weeks. Subtyping of the produced antibodies revealed a predominantly Th1-like response which did not change significantly with the absence or presence of particles. The particles likely function as an attractant for antigen-presenting cells (APCs), and probably do not affect EP or antigen expression to a significant extent. We conclude that micron-size gold particles injected intradermally together with DNA followed by EP give rise to an accelerated, potent immune response with a strong cellular component. This method may become important for the development of fast-acting therapeutic and prophylactic vaccines.

Keywords: Skin; DNA vaccine; Particulate adjuvant; In vivo electroporation; Noninvasive; Cancer

1. Introduction

DNA vaccination has become the fastest growing field in vaccine technology following reports at the beginning of the 1990s that intramuscular injection of plasmid DNA induces an immune response to plasmid-encoded antigens [1,2]. Plasmid DNA vaccines are nonpathogenic, stable at ambient temperatures, economical to produce, allow repeated immunizations, and frequently give rise to epitopes not present in conventional vaccines. However, there is a continuing need to make DNA vaccines more effective by improving intracellular DNA delivery and employing novel adjuvants. Various strategies to improve immune responses, including various DNA delivery techniques, have recently been reviewed [3,4].

Because of its unique immunological features, the skin appears to be an ideal target tissue for DNA immunization. Skin contains numerous and readily accessible bone marrow derived Langerhans cells (LCs) and dermal dendritic cells (DCs), potent antigen-presenting cells (APCs) critical to an effective immune response [5]. Moreover, keratinocytes within the epidermis are capable of efficiently synthesizing and secreting plasmid-encoded antigens. To make effective use of these inherent advantages for the development of potent skin-targeted DNA vaccines, several physical methods for the enhancement of DNA delivery into skin cells have been explored. Examples include the delivery of a hepatitis B DNA vaccine coated onto gold particles via gene gun to humans [6], needle-free vaccine injection using a high-pressure fluid jet [7], and the use of a microneedle array for delivering DNA vaccines into the epidermis [8]. Electroporation (EP), the application of short electrical pulses to the target tissue, renders the cell membrane transiently permeable to DNA and other molecules. This method has demonstrated its effectiveness as a nonviral gene delivery tool and its capacity for enhancing the potency of DNA vaccines (for the latest review, see

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Ref. [9]). Electroporation has frequently and successfully been used to augment muscle-targeted DNA vaccines (summarized in Ref. [10]) but only a few reports have addressed the use of EP for enhancing skin-targeted immunization [11,12].

The studies described here aimed at improving the effectiveness of DNA vaccination by combining enhanced intracellular delivery of DNA by EP with the administration of a particulate adjuvant, micron-size gold particles. It has been shown earlier that the injection of DNA bound to polymer particles evoked a much stronger immune response than the same amount of DNA in solution [13]. In our case, the uncharged gold particles were simply suspended in the DNA solution and the suspension was injected intradermally into mice. The DNA was not bound to, or adsorbed onto, the gold particles. DNA and particle injection was followed by EP with a user-friendly noninvasive meander electrode placed on the surface of the skin at the injection site [14,15]. The antibody titers and antibody subtypes obtained in the presence and absence of gold particles and EP, respectively, were determined. Administration of the gold particles accelerated the immune response and increased the percentage of responding animals. The observed increase and acceleration of the immune response by EP and gold particles encourages further development of this method for prophylactic and therapeutic vaccines against infectious agents, as well as diseases amenable to prevention or treatment by immune therapy.

2. Experimental

2.1. Plasmid DNA

Plasmid pHBsAg expressing the hepatitis B virus surface antigen (HBsAg) under the control of the human elongation factor 1α promoter was described previously [16]. Plasmid DNAs were purified using the Endo Free Plasmid Maxi Kit (Qiagen, Chatsworth, CA) and diluted with phosphate-buffered saline (1X PBS, Irvine Scientific, Irvine, CA) prior to injection.

2.2. Gold particles

Uncharged gold particles (1.6 μm diameter, BioRad, Hercules, CA) were mixed with plasmid DNA solution prior to injection (0.5 mg per site) to form a homogeneous suspension. Under the conditions used, DNA does not chemically react with, or adsorb to, the gold particles.

2.3. Animals

Female Balb/c mice, 6–10 weeks of age, were purchased from Harlan Sprague–Dawley (Indianapolis, IN) and housed at Genetronics (San Diego, CA) in accordance with the Guide for the Care and Use of Laboratory Animals. The studies described here were approved by the Institutional Animal Care and Use Committee (IACUC) of the study site. Mice were anesthetized by isoflurane inhalation. The fur around the lower dorsal area was shaved for immunization and placement of EP electrodes. All animals were humanely euthanized by CO_2 asphyxiation at the end of the experiments.

2.4. DNA immunization and in vivo electroporation

Immunization of four cohorts ($n = 6$ each) was performed by intradermal injection of pHBsAg plasmid DNA (10 μg in 25 μl per site, two sites per mouse) with a 28-gauge needle. Cohort 1 received DNA, and cohort 2 received a mixture of DNA and gold particles (no EP). Cohorts 3 and 4 were also injected with DNA and a mixture of DNA and gold particles, respectively; however, immediately after injection, a meander electrode (Genetronics, San Diego, CA) [14] was applied directly onto the skin, covering the area surrounding both injection sites. EP pulses (3 unipolar pulses at 5 Hz, 10 ms each, 70 V) were delivered by an ECM 830 square wave pulse generator (BTX, San Diego, CA). Each cohort received two booster immunizations identical to the primary immunization at the end of weeks 4 and 8, respectively. The animals were bled 2, 4, 6, 8, and 10 weeks after primary immunization and antibody titers were determined.

2.5. Measurement of antibody response and antibody isotypes

Titers of antibodies against HBsAg were determined in serum samples of individual animals (not pooled) using an AUSAB EIA clinical diagnostic kit, with quantification panel (Abbott Laboratories, Abbott Park, IL). IgG1, IgG2a, and IgG2b were determined in the same serum samples by standard EIA kits (Southern Biotech., Birmingham, AL). Sera diluted 1:50, 1:500, 1:1000, 1:2500, 1:5000, and 1:50,000 were assayed.

3. Results and discussion

Four cohorts of Balb/c mice were immunized as described in Section 2.5. The vaccine DNA used for this study, pHBsAg, encodes the ‘‘small’’ surface antigen of the hepatitis B virus, a well-characterized, relevant antigen that is frequently used in evaluating new methods of immunization. Table 1 shows the percentage of animals of each cohort that produced an antibody response. Mice immunized with DNA alone (cohort 1) did not respond at all, while animals vaccinated with DNA mixed with particles (cohort 2) responded only weakly, despite repeated boosting. On the other hand, animals that received EP after DNA ± particle injection (cohorts 3 and 4) responded at a high rate. Cohort 4, which received a combination of DNA, gold particles and EP, showed the fastest increase in the percentage of respond-

Table 1
Percentage of animals testing positive for anti-HBsAg antibodies

Cohort	Week 4	Week 6	Week 8	Week 10
1 DNA	0	0	0	0
2 DNA/gold	0	17	17	50
3 DNA/EP	33	67	83	100
4 DNA/gold/EP	50	83	100	100

Four cohorts of mice ($n=6$) were immunized with pHBsAg DNA and, as indicated, with or without gold particles ("gold") suspended in the DNA solution. Cohorts 3 and 4 received electroporation pulses (EP) at the vaccination site immediately after DNA ± gold particle injection. Identical boost immunizations were administered at the end of weeks 4 and 8. The percent of animals in each cohort testing positive for HBsAg antibodies is shown.

ing animals, reaching 100% at week 8. Cohort 3 (which received DNA and EP but no gold particles) consistently lagged behind cohort 4 by about 2 weeks. There is a clear difference in response rates between cohorts that were, or were not, electroporated (cohorts 3 and 4 vs. cohorts 1 and 2, respectively; $P<0.05$). Considering previously reported results [11,12,14], the stimulatory effect of EP was anticipated. However, the increase in response rate above the one achieved with EP, which can be attributed to the administration of particles, has not been described before (cohort 3 vs. cohort 4).

The mean antibody titers of cohorts 1–4 are shown in Fig. 1. Both the rate of increase and the level of the antibody titers at various time points parallel the percentage of responders shown in Table 1. In the absence of EP, naked DNA injection alone did not result in detectable quantities of antibodies against HBsAg, whereas the administration of gold particles did elicit a modest but measurable antibody titer (cohorts 1 and 2, respectively). In the presence of EP, gold particles accelerated the increase in antibody titers but did not affect the maximum antibody titer achieved (cohorts 3 and 4). The arithmetic mean antibody titer of cohort 4 at week 6 was 44 mIU/ml compared to the titer of 8 mIU/ml of cohort 3. This result demonstrates that in the presence of gold particles, antibody levels of potential therapeutic significance are reached approximately 2 weeks earlier than without gold particles. Up to week 6, the antibody titers achieved by a combination of EP and particles (cohort 4) are higher than the sum of the titers achieved by the separate administration of particles and EP, respectively (cohorts 2 and 3). With the DNA dose used in the experiments described, the difference in the immune response between cohorts 1 and 4 is remarkable and demonstrates the increase in efficacy that can be achieved over naked DNA injection by employing EP and a particulate adjuvant.

In mice, the extent of the cellular (Th1-type) response is indicated by the level of IgG2-type antibodies, while IgG1 antibodies are predictors of a primarily humoral (Th2-type) response. We and our collaborators [10,12,17] have reported earlier that intramuscular DNA vaccination followed by EP elicits a Th1-dominant response. Others found that DNA immunization with DNA-coated gold particles propelled into the upper layer of the skin by a gene gun device yields a Th2-type response, or a mixed Th1/Th2 response [18]. In order to obtain information as to the quality of the immune response elicited by our use of gold particles ± EP, we determined the relative quantities of IgG1, IgG2a, and IgG2b. The majority of responding animals had IgG1/IgG2a ratios <1, suggesting a primarily Th1-like response. However, the IgG1/IgG2a ratio for some animals of cohort 4 changed to >1 in weeks 6 and 10 (following boost one and

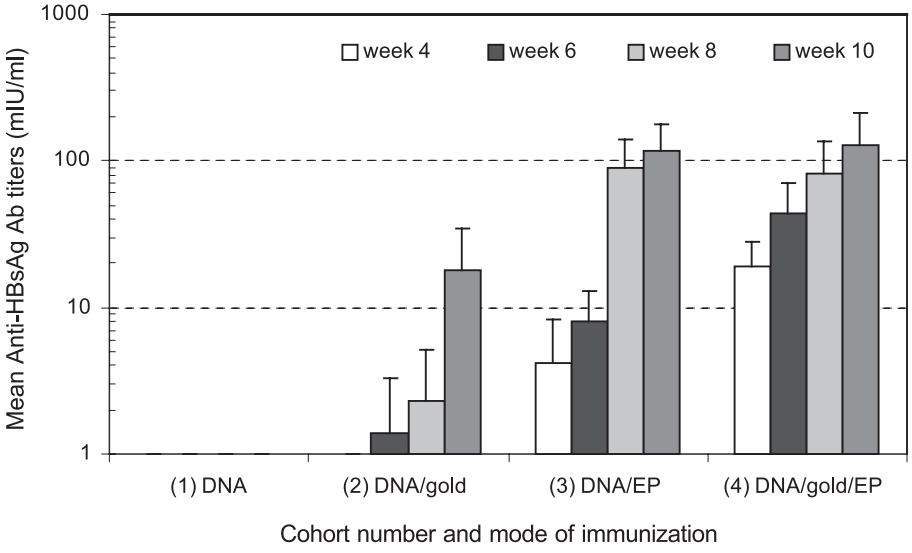

Fig. 1. Balb/c mice were injected intradermally with pHBsAg DNA. For cohorts 2 and 4, gold particles ("gold") were suspended in the DNA solution. Cohorts 3 and 4 received electroporation pulses (EP) at the injection sites immediately following DNA ± particle injection. At the end of weeks 4 and 8, boost immunizations identical to the primary immunization were administered. Antibody titers were determined by Abbott clinical ELISA at various times after primary immunization. Antibody titers are presented on a logarithmic scale as the arithmetic meanf ± S.E.M. using an unpaired one-tail Student's *t*-test ($n=6$).

two, respectively), indicating a mixed Th1/Th2 response. Our results are consistent with results obtained by Drabick et al. [11] who used intradermal DNA injection and EP with needle array electrodes.

The induction of a strong cellular response was confirmed by challenging animals immunized by the methods used for cohorts 1–4 (Fig. 1) by subcutaneous injection of tumorigenic CT26 cells, a murine colon adenocarcinoma cell line [19]. Immunized animals, in particular those which received EP, gold particles, or both, were largely protected from developing tumors when challenged with CT26 cells engineered to express HBsAg as a surrogate tumor antigen. However, nonimmunized animals, or immunized animals challenged with CT26 wild-type cells, rapidly succumbed to growing tumors (data not shown).

It is interesting to compare the results obtained with cutaneous vaccination presented here with results from intramuscular DNA vaccination obtained in the presence or absence of EP and gold particles, respectively [10]. In both cases, a predominantly Th1-like response has been observed. However, under the experimental conditions employed, the cutaneous route produced lower antibody titers, but a more prominent cellular response (as judged by protection against tumor challenge) than the intramuscular route.

It is now well established that EP, upon intramuscular injection of DNA vaccines, accelerates as well as increases the humoral and cellular response in small and large animals [9,10,16,17]. This is likely due to increased antigen expression, a consequence of EP-enhanced transfection, as well as to a transient inflammatory response induced by EP [17]. As we have shown in this study, gold particles injected together with DNA accelerate the immune response beyond the degree of acceleration achieved by EP. One possible mechanism underlying this phenomenon may be a direct positive effect of the gold particles on the efficiency of EP, which may further elevate antigen production. However, we have presented evidence elsewhere that gold particles are unlikely to significantly affect EP and, at least in muscle, gene expression is not enhanced by administration of the particles [10]. More likely, the observed enhancement and acceleration of the immune response in the presence of gold particles is due to the attraction of APCs, as suggested by results published by Singh et al. [13]. It is intriguing that gold particles enable an immune response in the absence of EP (Fig. 1, cohorts 1 and 2), whereas in the presence of EP, gold particles accelerate the immune response, but do not significantly affect the antibody titers reached at weeks 8 and 10 (Fig. 1, cohorts 3 and 4). It will be interesting to find out to what extent this behavior holds true for different DNA doses. Clearly, more studies are required to elucidate the mechanism and maximize the effect of particulate adjuvants on the desired immune response.

The described acceleration and/or increase of the immune response with EP and gold particles may become especially important for the development of vaccines where rapid responses are essential, e.g., for the treatment of acute infections and diseases, and/or the rapid prophylactic vaccination against infectious agents, including countermeasures against acts or threats of bioterrorism. Electroporation and particulate adjuvants may also allow the development of vaccines against weakly antigenic organisms, such as HIV and malaria, and contribute to the development of novel vaccines against chronic diseases, such as cancer, diabetes and arteriosclerosis.

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