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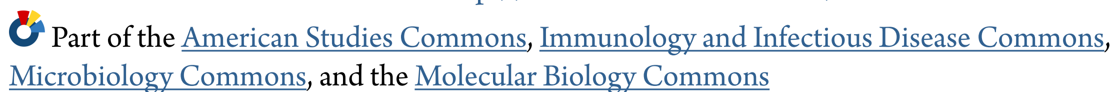

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Development of a Non-Invasive Electrode for Intradermal Electrically Mediated DNA Vaccination

by

Amy Lynn Donate

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Molecular Medicine
College of Medicine
University of South Florida

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Keywords: Electroporation, DNA Vaccine, Anthrax, *Bacillus anthracis*,
Hepatits B Virus

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Dedication

This dissertation is dedicated to my grandmother, Lorraine McConaghy, who always believed in me and were she with us today would have wanted a copy of this dissertation for everyone she knew. I miss you everyday, and I will always keep striving to do my best.

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List of Abbreviations

4PE: .... Four-Plate electrode

AAALAC: ... Association for the Assessment and Accreditation of Laboratory Animal Care

ANOVA: .... One Way Analysis of Variance

AP: .... Alkaline Phosphatase

APC: .... Antigen Presenting Cell

BA: .... *Bacillus anthracis*

BSA: .... Bovine Serum Albumin

CMI: .... Cell Mediated Immunity

CMV: .... Cytomegalovirus

CO_2: .... Carbon Dioxide

CpG ODN: . CpG Oligodeoxynucleotide

DAPI: .... 4′,6-diamidino-2-phenylindole

DC: .... Dendritic Cell

DMEM: ...... Dulbecco's Modified Eagle Medium

DMSO: ...... Dimethyl Sulfoxide

DNA: .... Deoxyribonucleic acid

DPGA: ...... poly-D-glutamic acid

EF: .... Edema Factor

ELISA: ...... Enzyme Linked Immunosorbent Assay

EP: .... Electroporation

FBS: .... Fetal Bovine Serum

FITC: .... Fluorescein Isothiocyanate

GFP: .... Green Fluorescent Protein

GMT .... Geometric Mean Titer

HBsAg: ..... Hepatitis B Surface Antigen

HBV: .... Hepatitis B Virus

HCV: .... Hepatitis C Virus

H & E: ...... Hematoxylin and Eosin

HEPES: ..... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV: .... Human Immunodeficiency Virus

HPV: .... Human Papiloma Virus

HRP: .... Horseradish Peroxidase

IACUC: ..... Institutional Animal Care and Use Committee

i.d.: .... Intradermal

I + EP: ..... Injection + Electroporation

IO: .... Injection only

i.m.: .... Intramuscular

i.p.: .... Intraperitoneal

JEV: .... Japanese Encephalitis Virus

LB: .... Lysogeny Broth

LCMV:....... Lymphocytic Choriomeningitis Virus

LF: .... Lethal Factor

LPS: .... Lipopolysaccharide

LTR: .... Long Terminal Repeats

MAPKK: .... Mitogen Activated Protein Kinase Kinase

MEA: .... Multielectrode Array

MHC: .... Major Histocompatibility Complex

μg: .... Microgram

mg: .... Milligram

MTT: .... 3-*(4,5-Dimethylthiazol-2-Yl)*-2,5-Diphenyltetrazolium Bromide

ng: .... Nanogram

NHP: .... Non-Human Primates

NPE: .... Non-Penetrating Electrode

PA: .... Protective Antigen

PBS: .... Phosphate Buffered Saline

PBST: .... Phosphate Buffered Saline + Tween 20

PE: .... Penetrating Electrode

Pen-Strep: Penicillin and Streptomycin

Pg: .... Picogram

pHBsAg: ... Hepatitis B Surface Antigen Plasmid

PHS: .... Public Health Service

pNPP: .... p-Nitrophenyl Phosphate

pPA: .... 1:1 Protective Antigen Plasmid

p/s/s: .... Photons/Second/Second (flux)

RSV: .... Respiratory Syncitial Virus

SARS-CoV: Severe Acute Respiratory Syndrome Virus

SV-40: ...... Simian Vacuolating Virus 40

TNA: .... Toxin Neutralization Assay

TNab: .... Toxin neutralizing antibodies

WNV: .... West Nile Virus

Abstract

Current progress in the development of vaccines has decreased the incidence of fatal and non-fatal infections and increased longevity. However, new technologies need to be developed to combat an emerging generation of infectious diseases. DNA vaccination has been demonstrated to have great potential for use against a wide variety of diseases. Alone, this vaccine technology does not generate a significant immune response for vaccination, but combined with delivery by electroporation (EP), can enhance plasmid expression and immunity against the expressed antigen. Most EP systems, while effective, can be invasive and painful making them less desirable for use in vaccination. Our lab recently developed a non-invasive electrode known as the multi-electrode array (MEA), which lies flat on the surface of the skin without penetrating the tissue. This study evaluated the use of the MEA for the development of DNA vaccines. We assessed the appropriate delivery conditions for gene expression and the development of humoral immunity. We used both *B. anthracis* and HBV as infectious models for our experiments. Our results indicated that the MEA can enhance gene expression in a mouse model

with minimal to no tissue damage. Optimal delivery conditions, based on generation of antibodies, were determined to be 125-175V/cm and 150ms with 200ug and a prime boost protocol administered on Day 0 and 14. Under these conditions, end-point titers of 20,000-25,000 were generated. Neutralizing antibodies were noted in 40-60% of animals.

Additionally, we utilized a guinea pig model to assess the translation potential of this electrode. The plasmid encoding HBsAg, pHBsAg, was delivered intradermally with the MEA to guinea pig skin. The results show increased protein expression resulting from plasmid delivery using the MEA as compared to injection alone. Within 48 hours of treatment, there was an influx of cellular infiltrate in the experimental groups. Humoral responses were also increased significantly in both duration and intensity as compared to the injection only groups. Results from both experimental models demonstrate that protective levels of humoral immunity can be generated and that this electrode should translate well to the clinic.

Introduction

Vaccine Development - history

The development of vaccines is arguably one of the most important medical advancements of the 20th century. However, humans have been attempting passive protection from disease since 500BC. The Chinese developed the first passive vaccines and since then our knowledge base has grown to allow us to develop more sophisticated technologies for fighting infectious disease. From the "black plague," to diseases like small pox, whooping cough, tuberculosis, measles, and influenza which at times decimated much of the world's population. The intentional development of vaccines became a reality when it was noted that milk maids exposed to cowpox did not become sick from small pox. This was the first modern recognition that passive protection from disease could be achieved and intentionally transferred. Prior to that Robert Koch developed his postulates based on the findings and identification of anthrax.

This led to a whole new era of vaccine development. The advent of the US Centers for Disease Control and Prevention was in direct response to help citizens understand their role in preventing disease.

Mass communications on posters and radio spread messages to keep your hands clean and stay indoors when sick. The mid-20th century gave rise to advancements in the area of bacteriology and infection disease. Additionally, the development technologies that would allow us to better diagnose and combat infectious diseases spurred advancements of vaccines against common agents like measles, mumps, and rubella, and whooping cough. Several of these vaccines were made mandatory by the US government to prevent further fatalities and spreading of the infections. The limitation that science is continually faced with is emerging infections where traditional methods of vaccinations have been unsuccessful. Development of vaccines has since sputtered but several methods have been developed that show promise for continued use against emerging infections

DNA Vaccines

DNA vaccines are genetically engineered plasmid DNA that encode for antigenic proteins under the control of a eukaryotic promoter. The most important parts of the plasmid are the promoter (most commonly CMV, RSV, SV40, and LTR used to drive constant stable expression in mammalian cells), the transgene (the gene of interest for vaccination), and the polyadenlyation sequence (responsible for mRNA stability and translation). The basic mechanism of DNA vaccination is that the DNA is injected into the tissue (muscle,

skin, subcutaneous space, etc) and enters the host cell. The DNA is translocated to the nucleus where transcription and translation occur to generate the target protein. The peptides are then presented on the cell surface by MHC I, or secreted (depending on the construction of the plasmid). The presented protein is then picked up and undergoes immune stimulation by APCs [2].

There are several advantages to using DNA vaccines over more traditional vaccination methods. First, DNA is highly stable, relatively easily produced, and stored [3-5]. Secondly, DNA vaccines can induce humoral and cellular immunity like live attenuated vaccines without the risk of reversion [6, 7]. Third, they have been demonstrated to have fewer side effects. Fourth, DNA vaccines can be multivalent, expressing multiple antigenic components on a single vaccine which could be useful for vaccinating against multiple agents simultaneously or agents multiple subunits of the same antigen [3-5]. Finally the use of DNA vaccines prevents the need for cold chain storage of vaccines during transport.

Initial studies into DNA vaccination began in the early 90's when Wolf et al demonstrated that DNA could be taken up by muscle cells and that integration into mouse genomic DNA did not occur [8]. These data opened up a whole new world for DNA vaccines to take off. Shortly thereafter several studies were conducted evaluating the

development of immunity from DNA vaccination. It was reported that mice injected with Influenza DNA encoding for the highly conserved nucleoprotein were protected from lethal challenge [9]. In this study both induction of humoral and cell mediated immunity was noted though protection was correlated to CMI. Since this study, several infectious agents have been tested for development of vaccination (Table 1). Many of these studies have been focused on viral pathogens like: HIV, HBV, HSV, LCMV and Rabies Virus. The use of DNA vaccination has also been evaluated for the development of immunity against parasitic infections like: *Plasmodium falciparum* and *Leishmania donovani*; as well as bacterial pathogens like: *Mycobacterium tuberculosis* and *Clostridium tetani*.

A variety of animal models have also been utilized to study DNA vaccination and the subsequent development of immunity. The most common has been the mouse model where initial studies were conducted as described earlier, but also continues to be the most common model for ease of use and reagent availability. However, several other models have been used including: cattle, rabbits, dogs, rats, guinea pigs, and NHP's with varying degrees of success.

Delivery of DNA is an important consideration development of immunity. The primary injection site for DNA vaccination has been muscle. This is primarily because early studies demonstrated that

direct injection into the muscle was superior to other tissue types [10] and that it was superior to adenoviral vectors [11]. This is most likely due to the long turnover of myocytes and their post mitotic state [12]. Despite the success of these results the preclinical models did not line up with initial human clinical trials [13]. For DNA vaccination to become a reality improved delivery systems were necessary to develop.

Table 1. DNA vaccines against infectious agents.

Viruses	**Bacteria**	**Parasites**
HIV [121, 122]	*Borrellia burgdorferi* [123, 124]	*Plasmodium falciparum* [125]
SARS [126-128]	*Clostridium tetani* [129]	*Leishmania major/ donovani* [130-132]
Influenza[133-135]	*Mycobacterium tuberculosis* [136-140]	*Toxoplasma gondii* [141-146]
Rabies Virus[147, 148]	*Bacillus anthracis* [149, 150]	*Tania Ovis* [151]
HBV [152-154]	*Clostridium botulinum* [155, 156]	*Schistosoma mansoni* [157, 158]
HCV [159, 160]		
Ebola Virus [161]		
HSV[162, 163]		
HPV [164]		
WNV [165]		
Rotavirus [166, 167]		
St. Louis Encephalitis Virus [168]		

*Pubmed keywords: DNA vaccine, Infectious disease

Electroporation

EP requires the application of electric fields causing permeabilization of the cell membranes [14, 15]. While there is still much unknown about the exact mechanisms of DNA entry into cell, what is understood is that it is a complex process involving interaction of the DNA with the cell membrane and that the DNA makes its way to the nucleus once inside the cell where it undergoes transcription and translation. At the time of EP pulsing, the DNA may be taken up by several processes, but one idea is that small “pores” are created in the membrane [15]. These holes are transient and are resolved very quickly; therefore, the DNA must be present at the time of pulsing. The resolution of the membrane pores allows the cell to continue to undergo normal cell function and processing including transcription/translation of the DNA.

Initial studies evaluating *in vivo* EP for transgene delivery and expression were performed on rat brain tumors [16] and rat livers [17]. Those studies demonstrated enhanced delivery and expression of plasmid DNA from EP mediated delivery. Further experimentation revealed that transgene expression could be increased 100-1000 fold from muscle EP stimulation [18-20]. Similar fold enhancements have also been seen in skin tissues ranging from 10-1000 fold [1]. Successful EP mediated DNA delivery has been demonstrated in most

tissue types and for several therapeutic and prophylactic indications such as cancer therapy, infectious diseases, wound healing, metabolic disorders and vaccines [21]. Recently several US clinical trials have been initiated. Eight clinical trials have been completed using EP, three assessing EP devices for use against infectious agents. 21 others are currently active or recruiting. 12 of those are involving DNA vaccination against infectious agents (clinicaltrials.gov; Keyword: Electroporation).

Electrically enhanced DNA vaccinations

Initial *in vivo* EP DNA vaccine studies evaluated gene expression and immune stimulation from delivery of plasmids encoding either HBV protein or HIV protein, gag, to the muscle. Their results confirmed that increased humoral responses to HBV [22] and cellular [23] immune response to HIV gag from EP compared to injection only (IO) of plasmid DNA. More recent studies have broadened the list of pathogens which EP has been successfully used *in vivo* to include other viral pathogens such as: HIV [24-27], SARS-CoV [28, 29], Influenza [30-34], WNV and JEV [35, 36], as well as HBV, HCV [37-41] and HPV [42, 43]. EP delivered DNA vaccines expressing proteins of the parasitic infection *Plasmodium falciparum*, one of the parasites causing malaria [44], as well as bacterial infections like *Bacillus anthracis* [45],

Clostridium botulinum [46], and *Mycobacterium tuberculosis* [47] have also been demonstrated to enhance immunogenicity. These results demonstrate the capacity of EP to enhance not only gene delivery and protein expression but also its ability to stimulate the host immune response against a wide variety of pathogens.

Target tissues for electrically mediated DNA Vaccine delivery

Currently, electrically mediated delivery of DNA vaccines typically employ painful invasive needle electrodes that are inserted into the muscle for electrical stimulation. The primary tissue used for *in vivo* EP is muscle because it is accessible, highly vascularized, multinucleated, and expresses DNA for long periods of time due to the post-mitotic nature of the tissue [12]. However, pain associated with administration is not desirable. As such, alternative delivery sites and methods have been explored. The skin is an attractive target for vaccination because of the high proportion of APC's and a large surface area. Recent studies, as well as work done in our laboratory, demonstrated that intradermal electrically mediated DNA expression can be increased both locally and systemically [48-53]. Electrodes developed for skin EP include: caliper, plate, tweezer, and clip electrodes as well as several needle electrodes [54-58].

Table 2. *In vivo* Electrically Mediated DNA vaccines against infectious

HIV/ SIV [23-27]
SARS [28, 29]
Influenza [30-34]
WNV [35]
JEV [36]
HBV and HCV [37-41]
HPV [42, 43]
Plasmodium falciparum [44]
Bacillus anthracis [45]
Clostridium botulinum [46]
Mycobacterium tuberculosis [47]

*Pubmed keywords: Electroporation and DNA vaccine or Electrically Mediated DNA vaccine.

The skin as a target for delivery

The skin is a highly complex and immunogenic organ. It is the largest organ in the body, and well equipped for recognizing and defending against infection. Its primary functions are to defend against infection and insulate and regulate temperature as well as regulating absorption and fluid loss and sensation. Human skin varies in thickness from about 0.5mm on the eyelids to 4mm on the hands and soles of the feet, with the majority of skin being between 1 and 2mm. The skin structure is made up of three layers: the epidermis, the dermis, and the subcutaneous layers. The epidermis consists primarily of keratinocytes, but also contains melanocytes and epidermal dendritic cells known as langerhans cells. It is made up of

five strata: stratum corneum, lucidum, granulosum, spinosum, basale. Cells are formed at the basale membrane and migrate up the strata changing shape and composition until they reach the stratum corneum where they are sloughed off. The rate of turnover is approximately 27 days [59]. The dermis, the main candidate for injection of DNA in the skin, consists of fibroblasts and dermal dendritic cells (highly efficient antigen presenting cells). In this layer, the hair follicles, sweat glands and blood vessels are found. The subcutaneous layer consists of connective tissue and fat. The primary cell types are fibroblasts, macrophages and fat cells [60].

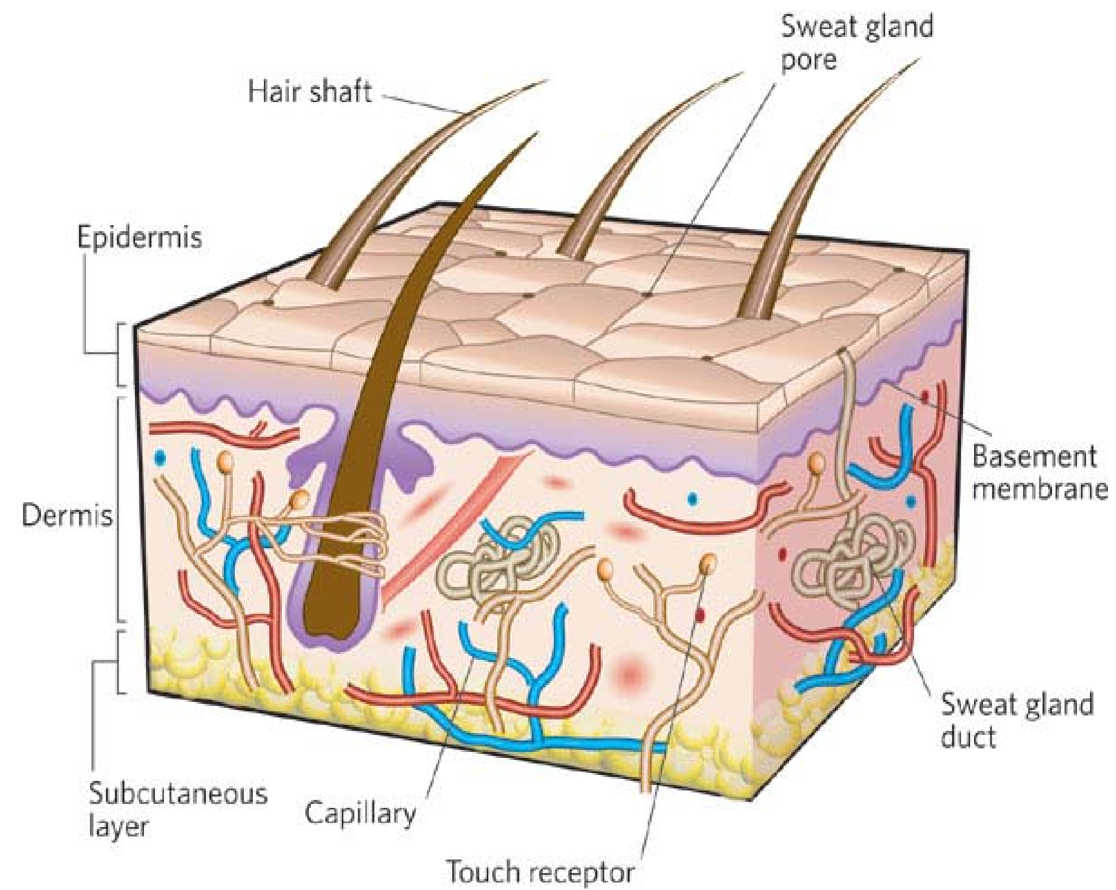


Figure 1. Human Skin Structure. Cartoon image of human skin, showing epidermis, dermis and subcutaneous layer. Also represented are hair follicles and the basement membrane. Image from www.skininfo.org.

Animal models for skin delivery

Several animals have been used for skin research including: mouse pigs, rabbits, guinea pigs, rats, and NHP's. The most common is the mouse, though this model has several disadvantages when compared to human skin. Mice have very thin skin, lots of hair follicles, and have a panniculus carnosus (layer of muscle beneath the dermis, not present in human skin). A better model for comparison to human skin is the guinea pig. Guinea pigs have langerhans cells in the epidermis, a dermal elastic fiber network, and contain fibroblasts, monocytes, and macrophages. A second model considered good for comparison to human skin is the pig Pigs have a thick epidermis, a spare hair coat, well differentiated papillary body in the dermis and elastic fiber network [61, 62]. An equally appropriate small rodent model is the Hairless guinea pig. This model in addition to having the same benefits as the traditional haired guinea pig also has a thick epidermis with distinct strata, serrated/non-serrated basal keratinocytes, a papillary dermal layer, and superficial microvasculature [63]

Electrode development for the skin

Several types of electrodes have been developed for use in the skin. These electrodes include: NPE's like plate, tweezer and caliper electrodes as well as PE and microneedle electrodes. Both types consist of single or multiple electrodes in various conformations intended to optimize plasmid delivery and expression [60].

Penetrating Electrodes

Penetrating skin electrodes consist of needle electrodes in various configurations. The PE's utilized in the skin were reviewed thoroughly by Gothelf et al [60] and included in Table 4 below. These electrodes range in electric fields (50-1800 V/cm), duration (50us to 650ms) and pulse number (1-18) depending on electrode design. The success of this type of electrode was recently published demonstrating the effective enhancement of transgene production in porcine skin [64]. Several PE's have been evaluated for the development of immunity against various infectious agents [60]. The most recent of these have demonstrated enhanced humoral and cell mediated immunity in comparison to DNA alone.

Non-Penetrating electrodes

NPE's have also been utilized for skin EP and provide the advantage of not being inserted into the skin. Early NPE's consisted primarily of two plated electrodes like caliper and tweezer electrodes that were squeezed to contact the skin surface. This generated variability in the distance between plates when applying pulses. While these electrodes were somewhat effective, experimentation revealed that DNA uptake and expression could be increased by applying electrical pulses in multiple directions. Therefore the Heller lab designed the 4PE. This electrode consisted of four plates with a non-conductive stopper that held the distance between the plates constant reducing variability when pulsing. The Heller lab found that expression using this electrode was significantly increased over IO and was consistent with other plate electrodes. However, due to the 6mm distance between the plates in this electrode the absolute voltage necessary for optimal expression generated pain.

Table 3. Published Non-Penetrating Electrodes.

Plate Electrode- Adjustable or Fixed Distance			
Reference	**Voltage**	**Duration**	**Number**
Titomirov 1991	400-600V/cm	100- 300μs	2
Zhang 1996	120V	10-20ms	3
Drabick 2001	1750V	100μs	6
Heller 2001	100V/cm	20ms	8
	1500V/cm	100μs	8
Lucas 2001	100V/cm	20ms	8
	1500V/cm	100μs	8
	750 + 14 + EEPV/cm	50μs + 20ms + 20ms	2 + 4 + 1
Maruyama 2001	12- 24V	50ms	8
Chesnoy 2002	200-400V/cm	20ms	10
Zhang 2002	50-100V	15-30ms	3 to 30
	75V	20ms	1 to 12
	100V/cm	2ms	60
Lee 2004	200-400V/cm	20ms	6
Medi 2005	50V	30ms	10
	100V/cm	10-30ms	5
	100-300V	10ms	5
Pavselj 2005	200V/cm	400ms	1
	250V/cm	20ms	6
	1000V/cm	100μs	1
	1750V/cm	100μs	6
	1000 + 140-200V/cm	100μs + 400ms	1 + 1
	700 + 200V/cm	100μs + 400ms	1 + 1
Thanaketpaisarn 2005	50-1000V/cm	5ms	12
Gao 2007	800V/cm	20ms	6
Heller 2007	100V/cm	2ms	8
Vandermeulen 2007	700 + 200V/cm	100μs + 400ms	1 + 1
Andre 2008	1000 + 80-200V/cm	100μs + 400ms	1 + 1
Vandermeulen 2009	700 + 200V/cm	100μs + 400ms	1 + 1
Gothelf 2011	1000 + 100V/cm	100μs + 400ms	1 + 1
Gothelf 2011	1000 + 80-160V/cm	100μs + 400ms	1 + 1

*Table continues on next page. Table reconstructed from Gothelf et al 2011 [60]

Table 3 cont.

Wires on skin- Custom built clips- Flat patches- MEA- 4PE			
Dujardin 2001	335V	0.5ms	10
	335V	5ms	10
	1000V/cm	100μs	10
Heller 2001	1500V/cm	100μs	8
Zhang 2002	75V	20ms	6
Babiuk 2003	60-80V	60ms	6
Heller 2007	10-1500V/cm	0.1-2000ms	8
Pedron-Mazoyer 2007	60-240V	20ms	8
Heller 2008	100V/cm	150ms	8
Mazeres 2009	60-240V	20ms	8
Heller 2009	100- 300V/cm	150-300ms	4

*Table reconstructed from Gothelf et al 2011 [60]

Table 4. Published Penetrating Electrodes.

Reference	Voltage	Duration	Number
Needle Electrodes- Needle Arrays			
Glasspool- Malone 2000	1750V/cm	100μs	6
Drabick 2001	1500V/cm	100μs	2 to 6
	1750V/cm	100μs	2 to 6
	2000V/cm	100μs	2 to 6
Byrnes 2004	200-400V/cm	20ms	6
	400-1800V/cm	20ms	6
	1750V/cm	100μs	6 to 18
Marti 2004	1800V/cm	100μs	6
Lin 2006	1800V/cm	100μs	6
Roos 2006	200V/cm	100μs	6
	275V/cm	10ms	8
	1125V/cm	50μs	2
	1750V/cm	100μs	6
	1125 + 275V/cm	50μs + 10ms	2 + 8
Zhao 2006	200V/cm	650ms	*
Kang 2008	50-250V/cm	100ms	6
Liu 2008	400V/cm	20ms	10
Brave 2009	1125 + 275V/cm	50μs + 10ms	2 + 8
Ferraro 2009	200V/cm	20ms	8
Lladser 2009	1125 + 275V/cm	50μs + 10ms	2 + 8
Roos 2009	1125 + 275V/cm	50μs + 10ms	2 + 8
Gothelf 2011	1000 + 8-140V/cm	100μs + 400ms	1 + 1
Needles Parallel to Skin Surface- Syringes- Plate and Fork Electrodes			
Maruyama 2001	12-50V	50ms	8
Lee 2004	50-200V/cm	20ms	6

*Table reconstructed from Gothelf et al 2011 [60]

Multielectrode Array

The MEA was designed to improve upon the 4PE. It is a 16 electrode array with 2mm spacing between electrodes. The reduced distance between electrodes decreases the absolute voltage applied while maintaining the electric Field (V/cm). For example, using the 4PE an electric field of 300V/cm would equate to applying an absolute voltage of 180 Volts (V=EF * (6/10)), whereas that same electric field would equate to an absolute voltage of 60 Volts (V=EF * (2/10)) using the MEA.

Our initial publications using the MEA to enhance gene expression demonstrated that the MEA was capable of inducing similar gene expression in guinea pigs and rats as conventional electrodes and that the level of expression was related to the duration and field strength applied[55, 65]. GFP results, demonstrate that expression was contained within the epidermis [1]. Muscle twitching from treatment was greatly reduced in both guinea pigs and rats [1, 55]. Finally, tissue damage from treatment was minimal and completely recoverable in 1 to 2 weeks [1].

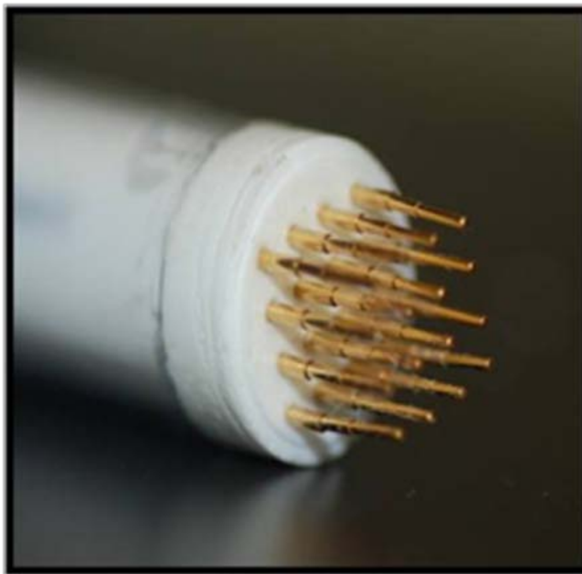


Figure 2. Non-invasive Multielectrode array. The MEA is designed with 16 electrodes spaced 2mm apart in a 4X4 square. The electrodes are round and gold plated with flat heads. [1]

Infectious Disease Models

Bacillus anthracis

Bacillus anthracis is a gram positive spore forming rod-shaped bacterium. *In vivo* the rods appear in short chains surrounded by a polypeptide capsule [66]. The Centers for Disease Control and Prevention classify *B. anthracis* as a category A agent because it "can be easily disseminated or transmitted from person to person, results in high mortality rates and have the potential for major public health impact, might cause public panic and social disruption, and requires special action for public health preparedness." It is found readily in soil and was historically a disease of livestock. Full virulence requires an anti-phagocytic capsule, and three toxin proteins.

Sporulation occurs from the presence of nutrient limited environment. In the case of *B. anthracis*, spores have been demonstrated to survive for decades and are demonstrated to aid in dissemination of bacterium. The spore structure is made up of five parts: the core, cortex, coat, innerspace, and the exosporium. The exosporium contains several proteins that may play a role in vaccination. One exosporium *B. anthracis* protein that has been extensively studied is Bacillus collagen like protein A. This protein has

been shown to function in mediating the specificity of *B. anthracis* spores to be taken up by macrophages [67, 68]

There are two toxins produced by *B. anthracis* that are of importance for clinical disease progression. They are edema toxin and lethal toxin. These binary toxins are comprised of protective antigen (PA) and either edema factor (EF) or lethal factor (LF) all produced after spore germination. The protective antigen protein is an 83Kda protein in its inactive state. It is cleaved by furin-like proteases to its 63Kda active [69]. Several active PA's come together to form either the heptameric or the highly stable octameric (common under normal physiologic pH and temperatures) prepore [70-72]. Multiple copies of the LF and EF bind to the PA prepore and are endocytosed and transferred into an acidic compartment. The PA prepore channel undergoes a conformation change and insert into the membrane forming a cation selective channel. The PA channel unfolds and using gradient that develops across the endosomal membrane translocates LF and EF into the cytosol [73-75]

Edema toxin is a calmodulin-dependent adenylate cyclase that alters water homeostasis causing edema and impairs neutrophil function, rendering the host further susceptible to infection [76]. Lethal toxin leads to the release of reactive oxygen intermediates as well as the production of proinflammatory cytokines tumor necrosis

factor and interleukin-1b responsible for rapid circulatory collapse leading to disruption of MAPKK pathways and cell death [77, 78].

Clinical disease

B. anthracis causes anthrax disease, originally known as wool-sorters, because it was those individuals that primarily acquired the disease from spore infested wool. Anthrax has three clinical manifestations. The first, cutaneous anthrax, acquired through a break in the skin, is usually self-limiting. This form of anthrax is estimated to account for greater than 90% of human anthrax cases in the world [79]. The second and slightly more severe is gastrointestinal, which is acquired through ingestion of infected meat. Mortality rates with antibiotic treatment are about 40% [66]. The final and most life threatening form, known as pulmonary anthrax, is caused by inhaling *B. anthracis* spores into the lungs. Upon inhalation spores are taken into the alveolar spaces and engulfed by alveolar macrophages. They are transferred to the lymph nodes, where germination occurs. Upon germination of spores, toxins are produced that lead to flu-like symptoms and progress to toxemia and death from shock and multi organ failure [78].

Anthrax Vaccine Adsorbed and novel vaccines

The current available vaccine is a recombinant protein vaccine delivered intramuscularly in a 5 regimen dose over the course of 18 months. Following this series, annual boosters are recommended [80]. The protein is isolated from a toxigenic non-encapsulated form of the bacterium V770-NPR1 [80-85]. Side effects have been noted in approximately one-third of vaccinated individuals including: injection site swelling, redness, and tenderness [86]. The primary component of this vaccine is PA [83]. Research demonstrates that some antibodies formed against PA can prevent toxin formation which is a critical component of vaccine development [87, 88]. For this reason most research conducted for the formulation of novel *B. anthracis* vaccines has utilized PA as a target.

These PA vaccines have been shown to have varying success upon challenge [3, 4, 80-82, 84, 85, 89-92]. Augmented rPA vaccines have been combined with CpG ODN, bacterial DNA fragments, *E. coli* LPS, complement receptors for targeting APC's, and complement C3d. Recombinant PA has been combined with various other *B. anthracis* components including inactivated spores, LF and or EF, capsule gamma DPGA, and unencapsulated spores. Some protection was shown from PA DNA vaccination but not against fully virulent strains unless combined with other agents [89]. Recently, a study evaluating

an IM EP mediated PA DNA vaccine was published. Their results demonstrated the value of this technology, by achieving survival in 4 of 5 NHP's against an aerosol challenge of *B. anthracis* [93].

Hepatitis B Virus

HBV is a member of the Hepadnaviradea family whose genome is made up of circular DNA. As its name suggests, this family of viruses causes infections of the liver. Liver disease from HBV can present in a variety of ways from fulminant hepatitis, cirrhosis, or hepatocellular carcinoma. Approximately 15-40% of chronic HBV sufferers will develop significant liver disease. The main components of this virus are: surface and core antigens, DNA polymerase, and an x antigen of unknown function [94]. There are 8 known genotypes of HBV denoted A through H [95].

The current vaccine for HBV is a recombinant protein vaccine derived in yeast. The protein used in this vaccine is recombinant HBsAg. This vaccine is a 3 course vaccine given to infants within the first two months after birth. Current data shows that vaccination with this vaccine lasts long term and that additional boosters are not necessary for properly vaccinated immunocompetent individuals [96].

For this reason, it is an appropriate candidate for comparison of our vaccine model. Established protective levels of anti-HBs titers are greater than 10mIU/ml.

Significance

The development of vaccines has been hampered by the emergence of infectious agents and lack of new techniques. Novel methods like DNA vaccination provide a useful alternative to traditional methods by providing ease of production, stability in transport, small amounts necessary. Unfortunately, DNA vaccines delivered directly have been ineffective and require the use of alternate delivery techniques. EP has been shown to effectively increase gene expression as well as humoral and CMI with DNA vaccination. Our model for DNA vaccination involves the use of a novel NPE, the MEA, for the induction of humoral immunity. This electrode lays flat on the skin's surface and is applied after intradermal injection. This method is far less invasive and is more "friendly" for the patient.

Additionally, when evaluating novel methods of DNA vaccination it is important to evaluate clinically relevant infectious models to determine the viability of your method. *B. anthracis* is the causative agent of anthrax and a potential threat for use as a bioweapon.

Finally, quite often when evaluating new methods appropriate animal models are sometimes not used and fail to appropriately determine what the possible effect will be in humans. We have utilized the guinea pig model to more accurately reflect the effect of the MEA on human skin. This study is significant because it not only demonstrates a novel method for DNA vaccination but also evaluates the effectiveness of this method for immunogenicity as well as translation to the clinic.

Goals and Objectives

Purpose

The purpose of this study was to assess the functionality of the MEA for use in DNA vaccination. In particular for the development of humoral immunity against the clinically relevant BA infectious model in mice as well as translational relevance by vaccination in a human like skin model. This study evaluates the effects of both plasmid and EP has on generation of humoral immunity as well as the effect of EP with the MEA on the skin.

Hypothesis

Electrically mediated DNA vaccination with the MEA will enhance immune stimulation against BAs in a mouse model and HBV in a human-like skin model, guinea pigs.

Specific Aims

Aim 1: Determine the effect of electrically mediated delivery with the Multi-Electrode Array on plasmid expression in mouse skin

a. Compare gene expression from electroporation with the Multi-Electrode Array at various field strengths to the optimized skin (4PE) and muscle (4 needle) electrodes.
b. Evaluate the tissue damage and inflammation caused by MEA mediated electroporation by histology and visual assessment.
c. Evaluate differences in the gene expression profile from MEA mediated EP at high and low electric fields.

Aim 2: Determine the effect of electrically mediated delivery with the MEA on immune stimulation against B. anthracis

Sub aim a: Purification of PA plasmid and confirmation of expression in vitro.

1. Plasmid purification and digestion
2. PA expression in vitro
3. MEA mediated DNA vaccination with PA plasmids induces anti-PA antibodies in vivo

Sub aim b: Determine the appropriate delivery conditions for induction of humoral immunity against *B. anthracis*.

1. Evaluate the effect of plasmid dose on MEA mediated humoral immune stimulation
2. Evaluate the effect of number of treatments on MEA mediated humoral immune stimulation
3. Evaluate the electric field effect of MEA mediated humoral immune stimulation

Sub aim c: Determine the *in vitro* protective potential of the optimized delivery conditions

Aim 3: Evaluate the potential for translation of electrically mediated delivery with the MEA in a human-like skin model.

a. Evaluate the effect of MEA mediated EP on human-like skin by histological analysis and visual assessment
b. Evaluate MEA mediated DNA vaccine expression in a human-like skin model.
c. Determine the humoral stimulation from MEA mediated DNA vaccination against HBV

Materials and Methods

General Methods

***Plasmid purification*.** Plasmid DNA was produced by transformation into *E. Coli* XL-10 gold cells. Transformed cells were plated on antibiotic resistant LB agar (Ampicillin 100ug/ml or Kanamycin 50ug/ml) and incubated overnight at 37°C. Colonies were picked and cultured in 2.5 Liters of antibiotic containing media. Plasmid was isolated using Qiagen plasmid Giga-prep kit per manufacturer's protocol. Briefly, cultures were spun down at 3000 rpm for 30 minutes at 4°C. The bacterial pellet was resuspended in 125 ml of Buffer P1. 125mls of Buffer P2 was added and inverted 5 times to mix and incubated for 5 minutes at room temperature. 125mls of Buffer P3 was added and mixture was added to the Qiafilter and allowed to incubate for 10 minutes. Mixture was vacuum filtered and 30mls of Buffer ER was added and incubated on ice for 30 minutes. Qiatip 1000 was equilibrated with 75mls Buffer QBT. Mixture was added to Qiatip to bind DNA. Tip was washed with 600mls Buffer QC. DNA was eluted with 100mls of Buffer QT. DNA precipitation was performed with 70mls of isopropanol and spun at 15000 x g for 30 minutes at 4°C.

DNA pellet was washed with 70% Ethanol and respun for 10 minutes. Pellet was air dried and resuspended at 2mg/ml in physiological saline.

Cell Lines and Complete Growth Medium*:* B16F10 cells were purchased from ATCC and grown in McCoy's 5A media supplemented with 10% FBS and Gentamycin. J774A.1 Macrophages were also purchased from ATCC and were grown in DMEM supplemented with 5% FBS, 10mM HEPES Buffer, and Pen-Strep.

DNA Digestion*:* PA plasmids were digested with restriction enzymes NotI or KpnI and incubated at 37°C for 1.5 hours. 6X loading dye was added to stop the digestion and run on a 1% agarose gel with HindIII lambda marker. The gel was run at 100V for 1 hour. The gel was incubated for 15 minutes in Ethidium Bromide and UV light used to visualize the resulting bands.

Mouse model methods

Ethics Statement*:* Animal procedures were conducted at either USF vivarium, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)

and the Public Health Service (PHS), or Old Dominion University Center for Bioelectrics' vivarium, which is currently undergoing AAALAC accreditation. Research was conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC) at both institutions (protocol # 10-006). All animals were housed, handled and utilized following guidelines of the United States National Institutes of Health.

Animals and injections: 6-8 week old female Balb/c mice were intradermally injected at two sites on the left flank with 50µl of plasmid for experimental animals. Experimental mice were boosted either once or twice 14 days after the previous treatment (Day 14, Day 28). All experiments included control animals of 10µg muscle injected recombinant protein as well as injection only. Recombinant protein injections were administered at Day 0, 14, and 28. Mice were bled by tail vein at various time-points. All animals were anesthetized with 2-3% isoflurane + O_2 for treatments.

Plasmids: The plasmids used for these experiments were pSecTagPA and pCMVER/PA at various concentrations for *B. anthracis* studies. The PA plasmids were generously donated by the Hahn lab (University

of Hoenheim, Germany). Reporter assays were done using pGwizLuc and pGwizGFP (Aldevron) also at 2mg/ml.

Electroporation*:* The MEA was used at applied electric fields ranging from 25 to 225V/cm but always maintained constant pulse duration and delay of 150ms. A sequence of 9 4X4 squares was applied 4 times for a total of 72 pulses. Electrodes were circular, gold plated and flat at the end with a 0.2mm diameter.

In vivo Bioluminescent Imaging: The Caliper life sciences IVIS Spectrum was used for live animal bioluminescent imaging. Animals were injected i.p with 15mg/ml luciferin. 20 minutes post luciferin injection the animals were imaged and relative light units measured. All luciferase data is represented as average total flux (photons/sec/sec) per injection site.

Tissue Collection and sectioning: Mouse skin was collected at various time points from 24-72 hours and up to 7 days after treatment. Mice were humanely euthanized using CO_2 asphyxiation. Tissue was marked at time of treatment to notate the region to be removed. Skin samples were immediately placed on dry ice or in

formalin for sectioning. Frozen skin sections were sliced using the Thermo Fisher cryostat 550 at -17C in OCT medium

Immunohistochemistry: Sections were placed on slides and fixed for twenty minutes in 75% Acetone and 25% Methanol and placed at -80°C until imaging. Slides were blocked for 1 hour in PBS with10% goat serum at room temperature in the dark. FITC conjugated goat anti-GFP antibody diluted 1:500 in blocking buffer was added overnight at 4°C. Slides were washed with PBS and Dapi added to visualize nuclei. Slides were imaged immediately to prevent loss of fluorescence.

Histology*:* Skin samples were taken from both mice for histological analysis. Mouse skin was collected 48 hours after treatment and fixed in formalin. H & E staining was performed to assess inflammation and damage.

Sandwich ELISA for PA detection: Anti-PA coating antibody (Abcam 18725) was diluted 5µg/ml in 50mM Sodium Carbonate. 100ul of coating buffer was added to each well and incubated overnight at 4°C.

The next day the plate was blocked with PBST + BSA for 2 hours at 37°C. Supernatants were added directly to each well with 100µl, lysates were lysed with NP40 buffer (100ml of 100mM Trizma pH 8.0, 50ml Glycerol, 5ml Triton X100, 4g NaCl, 10ml of 100mM EDTA pH 7.4, diH2O) for 30 minutes on ice. PA antigen (List Biologicals 171B) was used as a control to quantitate amount of PA present. PA was diluted to 10000ng/ml and 100µl added to each well for standards. Four fold dilutions were made to generate a standard curve. Samples were incubated for 2 hours at 37°C. Wells were washed with PBST 5 times. Secondary was (Abcam 18723) added for 1 hour at room temperature. Secondary was diluted to 1ug/ml and 50ul added to each well. AP conjugated antibody was added for 1hr in dark at room temperature. To colorize, pNPP (Sigma) was added and the plate was read at 405nm.

***Indirect ELISA for the determination of antibodies**:* Briefly, antigen was coated at 0.1 to 1µg/well and incubated overnight at 4°C. Plates were blocked with either BSA-PBST (anti-HBs) or 5% skim milk buffer (anti-PA) for 2 hours at 37°C. Samples were diluted in blocking buffer and incubated for 1 hour at 37°C. HRP conjugated secondary antibodies (Santa Cruz) were diluted in blocking buffer to working concentration and added for 30 to 60 minutes in the dark. R&D

substrate was added for 10 minutes and stopped with 2N H_2SO_4. Plates were read at 450nm and results represented as mean titers.

***Toxin neutralization assay**:* 50,000 cells/well of J774A.1 murine macrophages were plated in 96 well cell culture plates. The next day serum was diluted starting at 1:50 in media and incubated for one hour with 100ng/ml protective antigen. Lethal Factor was added to the Serum/Protective antigen mix at a final concentration of 80ng/ml. Media was removed from the cells and the serum/PA/LF mix was added to the macrophages for 4 hours at 37 and 5% CO2. All plates contained a titration curve to confirm that the concentration of toxin used was sufficient to cause 95% cell death. Following the 4 hour incubation, 25µl of MTT (5mg/ml) was added and incubated for an additional 2 hours. Media was removed by vacuum suction and 100µl of DMSO was added to break up crystal formation. Plate was read at 560nm.

Guinea Pig model methods

***Ethics Statement**:* All animal procedures were conducted the University of South Florida, College of Medicine vivarium which is fully accredited by AAALAC and the PHS. Research was conducted under a

protocol approved by the IACUC at the University of South Florida, College of Medicine (protocol # 2879). All animals were housed, handled and utilizing following guidelines of the United States National Institutes of Health.

Animals: Female Hartley guinea pigs between 200-250g were used in this study to evaluate skin EP conditions. Guinea pigs were housed at the University of South Florida, College of Medicine vivarium and were rested for one week prior to experimentation. Guinea pigs were anesthetized with 2.5-3.0% isoflurane before and during all procedures. No previous exposure to HBV was known.

Plasmid: The plasmid used in this study was gWiz™ HBsAg (Aldevron, Fargo, ND). This plasmid encodes for the surface antigen of Hepatitis B and is driven by the CMV promoter.

***Immunization**:* All guinea pigs were intradermally injected with 100μg (2mg/ml) of gWiz™ HBsAg at two sites on the left flank. MEA EP was performed at 300V/cm and 150ms and 72 pulses. The two groups used in this study were control group injection of plasmid only

(IO) and injection of plasmid plus EP (I +EP). All groups were boosted with the same condition at Day 14.

Serum collection: Guinea pigs were anesthetized with 2.5-3.0% isoflurane. Blood was collected from the jugular vein at various time points from Day 0 through Day 168. Blood was collected and serum isolated in serum separator tubes. Serum was diluted two-fold starting at 1:10.

Tissue collection*:* Guinea pigs were treated as described with gWiz™ HBsAg with and without EP. Those guinea pigs whose tissue was collected for plasmid expression were sacrificed 48 hours after one treatment and skin samples were harvested by excising the treatment site and followed by freeing. Those guinea pigs whose tissue was collected to assess damage and cell infiltrate were treated and harvested 96 hours after one treatment and the tissue was snap frozen.

Indirect ELISA for the detection of Hepatitis B surface antigen antibodies: An ELISA was used to assess the production of antibodies

from treatment and performed per manufacturer's protocol (Aldevron). Briefly, a 96-well plate (Nunc) was coated with 10μg/ml of HBsAg (Aldevron) and allowed to coat overnight at 4°C. The plate was blocked with 3% BSA in PBST for 2 hours at 37°C. Serum samples were two-fold diluted in blocking buffer and added to the plate for 2 hours at 37°C. Goat anti-Guinea pig-AP antibody was added at a 1:10000 dilution in blocking buffer. AP substrate, pNPP, (Sigma) was added to colorize and the plate was read at 405nm.

Immunohistochemistry: An anti-HBsAg was used to detect plasmid expression. Skin samples taken 48 hours after treatment were frozen, sectioned, and placed on slides. Slides were rehydrated and then blocked with 3% BSA in PBST and incubated in a humidifying chamber for 1 hr. A HRP conjugated anti-HBsAg (AbD Serotec) was made in blocking buffer at a 1:200 dilution. All samples were counterstained with H & E.

Histology: Samples collected at 96 hours frozen, sectioned, and placed on slides were stained with H & E to determine the extent of cellular infiltrate/inflammation.

Statistical analysis: All Guinea pigs were bled at Day 0 to determine background optical density (OD). OD's were averaged and 2 standard deviations added to determine positive (0.1 OD). Experimental serum samples were diluted two-fold starting at 1:10. End point titers were calculated and plotted as Geometric Means. Significance was determined by student t-test using the bonferroni correction for multiple comparisons.

Results

Aim 1: Determine the effect of electrically mediated delivery with the MEA on plasmid expression in mouse skin.

Introduction:

EP has been demonstrated to be an effective delivery platform for DNA. However, it is limited in its use due to the current electrode designs. Currently those electrodes require either high voltages that would not be tolerable for human use or PE's that involve insertion into the tissue. We have designed a novel electrode that both eliminates penetration of the electrode as well as reduces the absolute voltage necessary for delivery. Here we establish that this devices elicits similar gene expression levels as the current devices with minimal to no damage.

a. Comparison of gene expression from EP with the MEA at various field strengths to the optimized skin (4PE) and muscle (4 needle) electrodes.

In order to assess the ability of the MEA to enhance gene expression in a mouse model, Balb/c mice were injected with 50ul of GwizLuc (2mg/ml) intradermally on the left flank. Sites were electroporated with various electric fields with the MEA or 100V/cm with the 4PE. A control group of injection of plasmid only was included

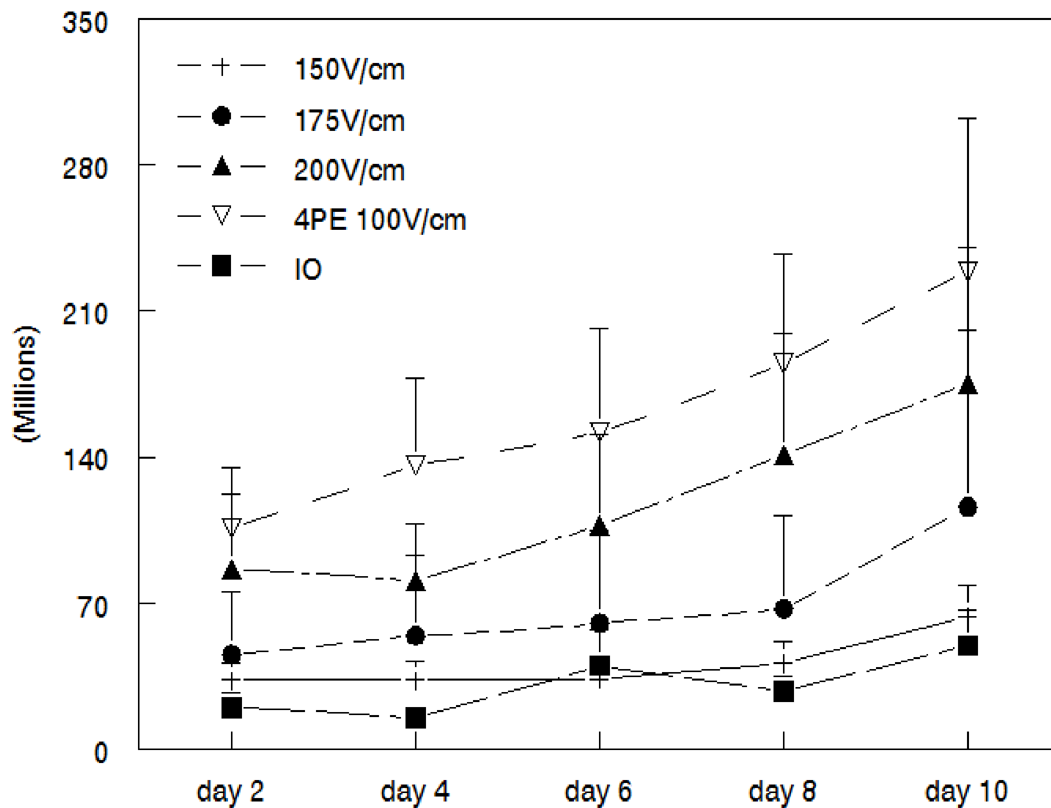


Figure 3. Luciferase Gene expression from MEA EP. GwizLuc plasmid (2mg/ml) was injected into the left flank of Balb/c mice. Treatment sites were either EP with the MEA or 4PE at specified electric fields. Control group of IO was also included.

(IO). Figure 3 shows that using the MEA, luciferase expression can be increased and that the increase is field dependent. Higher electric fields result in increased luciferase expression. However, all MEA conditions are greater than IO and demonstrate similar expression patterns as the control 4PE animals over time. Visual tissue damage was seen in animals treated with the MEA at 200V/cm. Though this condition represented the highest level of gene expression with the MEA conditions above 175V/cm will not be used to prevent potential tissue damage.

b. Evaluate tissue damage and inflammation caused by MEA mediated EP by histology and visual assessment.

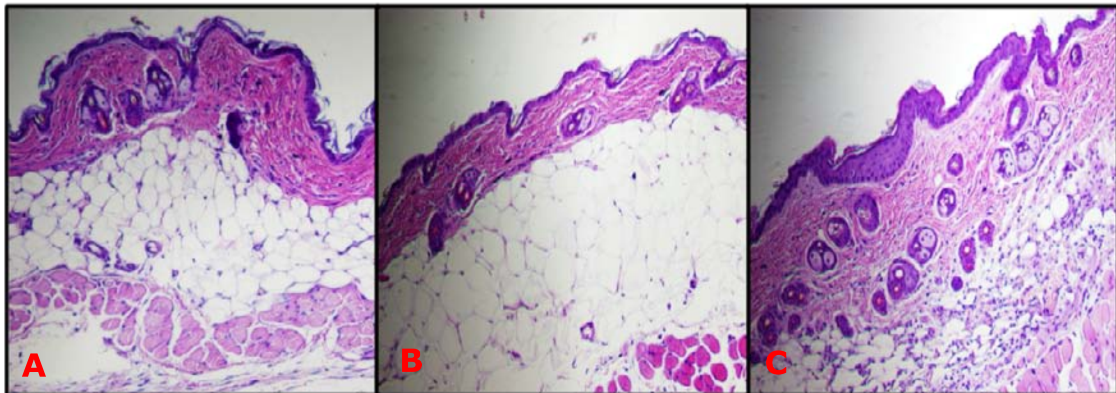


Figure 4. Effect of MEA mediated EP on mouse skin histology. Balb/c mice were injected with plasmid DNA and EP to assess changes in skin from treatment. Samples were collected 48 hours after treatment. A) No treatment. B) IO of plasmid DNA. C) Injection and EP with the MEA at 175V/cm.

Histology was performed to evaluate the skin tissue for damage at 175V/cm. IO samples show no gross visual difference in swelling or skin damage from EP treated animals. Histologically, however, (figure 4 a & b) EP samples showed a large influx of cellular infiltrate

c. Evaluate the differences in DNA uptake from MEA mediated EP at high and low electric fields

Expression of plasmid DNA at high and low electric fields was evaluated using GFP. The use of GFP instead of Luciferase allows us to visualize the location and number of cells expressing the protein as opposed to total expression. Here we were able to evaluate whether these electric fields have different DNA distribution after EP and subsequent differences in expression. Figure 5, shows MEA EP at 125V/cm and MEA EP 175V/cm for both 24 and 48 hours after EP. Enhanced expression from EP can be seen in both 125 and 175V/cm conditions at different time points. EP with 125V/cm demonstrates the highest quality expression. EP with 125V/cm has good distribution of expression along the epidermis and is more pronounced at 48 hours.

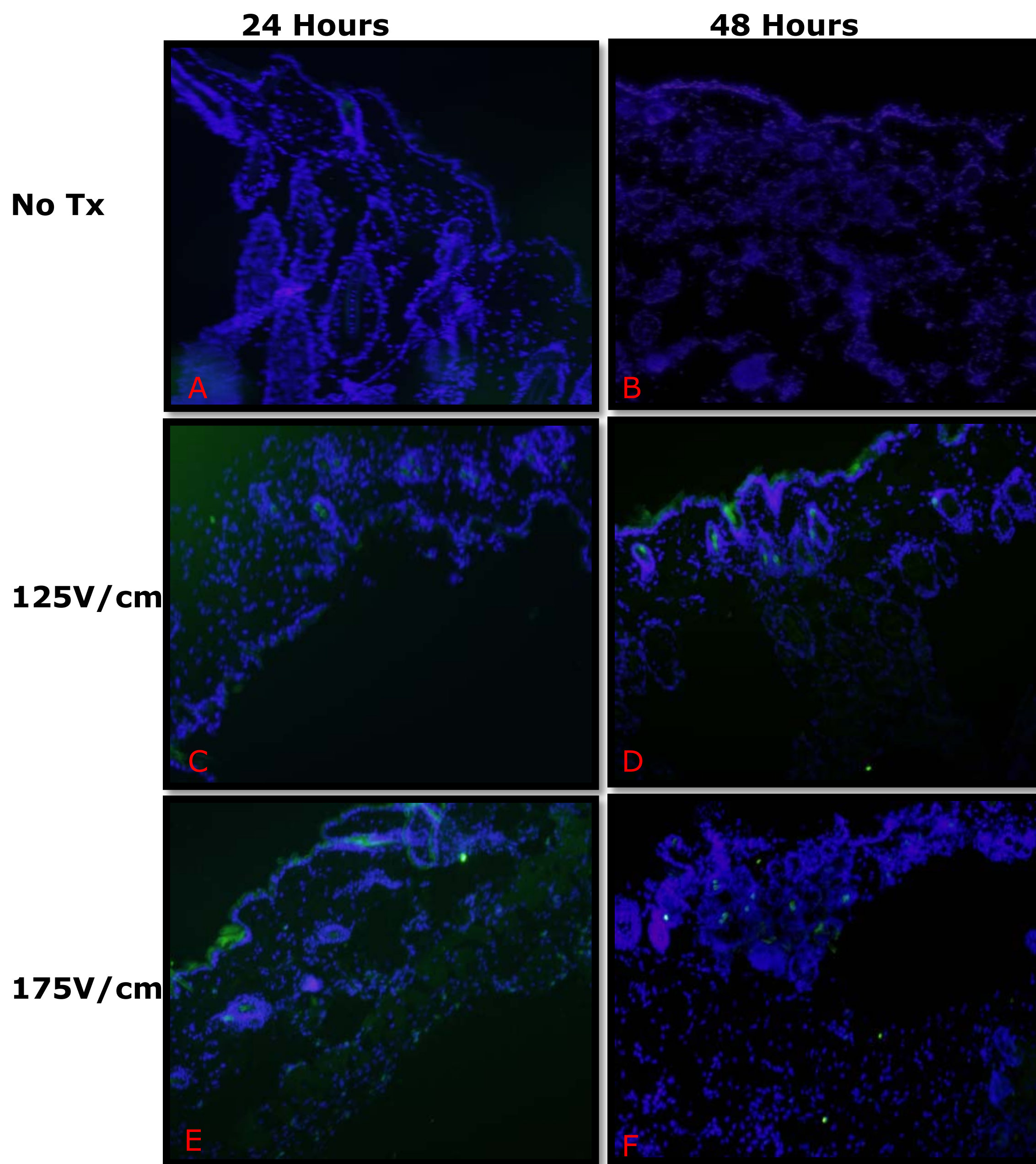


Figure 5. GFP expression from MEA mediated EP at various conditions. Mice were injected with pGwizGFP plasmid (C-E) and EP at either 125V/cm (C and D) or 175V/cm (E and F). Skin was collected and snap frozen at 24 (A, C, and E) and 48 (B, D, and F) hours after treatment.

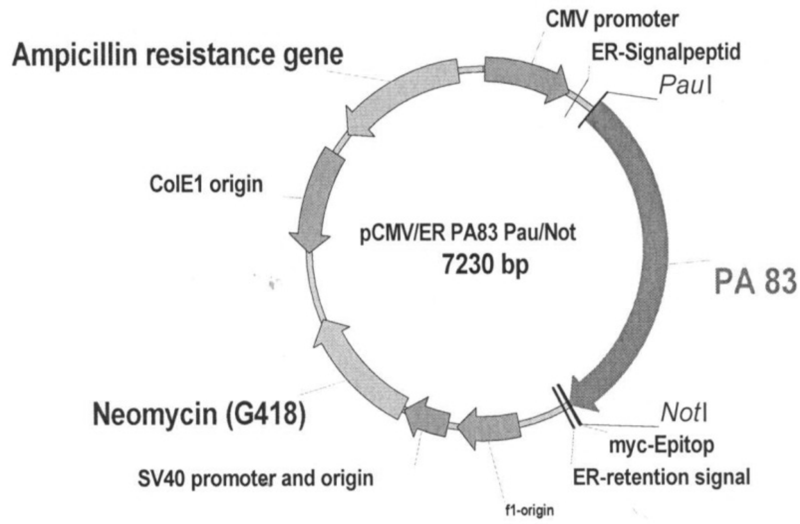
Aim 2: Determine the effect of electrically mediated delivery with the MEA on immune stimulation against B. anthracis.

Introduction

B. anthracis is of clinical relevance as a bioterrorism weapon. The current available vaccine has several side effects that range from mild to severe and requires several initial vaccinations followed by annual boosters. The vaccine is a recombinant protein vaccine and therefore a good candidate for development of a new vaccine model. Our group, and others, proposed the use of DNA vaccines to generate lasting immunity against this threat. In 2004, the Hahn group constructed two plasmids expressing full length PA. These plasmids were designed to secrete PA (pSecTagPA; Fig 6A) and bind to the membrane (pCMVER/PA; Fig 6B).

Their results demonstrated that these plasmids when delivered by the gene gun could generate humoral immunity including antibodies against neutralizing epitopes of PA. Here we utilize these established plasmids to determine whether EP with the MEA can generate humoral and neutralizing immunity against *B. anthracis* and those conditions of the MEA that are best suited for developing immunity.

A



B

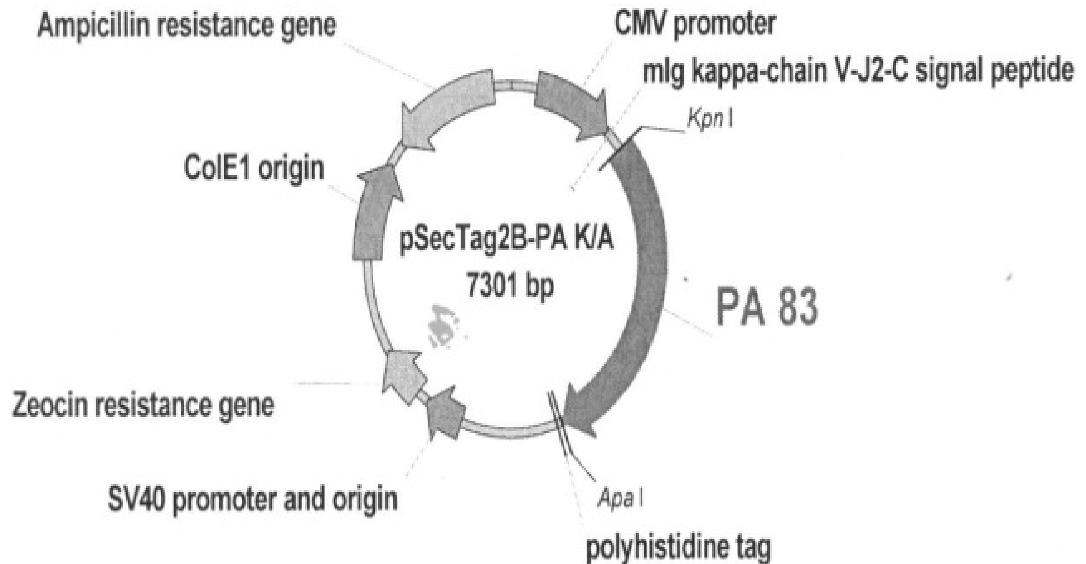


Figure 6 Protective antigen plasmid constructs. Construction of the PA plasmids used in this study was done by the Hahn lab and published in 2004 in Vaccine. Both plasmid backbones were commercially made by Invitrogen and are designed to express full length PA under control of the CMV promoter A) pCMVER/PA contains a sequence for targeting expression to the endoplasmic reticulum. B) pSecTagPA contains a secretion sequence.

Sub aim a: Confirmation of expression of PA plasmids in vitro and in vivo

1. PA plasmid preparation and isolation

PA plasmids were prepared as described in Methods and digested with NotI (pCMVER/PA) and KpnI (pSecTagPA). The resulting digests, shown in figure 7, show undigested and digested plasmid. Lane 1 is the HindIII lambda marker. Lanes 2 and 3 are undigested and digested pCMVER/PA respectively. Lanes 4 and 5 are undigested and digested pSecTagPA respectively. The bands for pCMVER/PA are seen at 7230bp corresponding to a correctly linearized plasmid. The band for pSecTagPA corresponds to 7299bp, again a correctly linearized plasmid. Both plasmids were purified cleanly and linearize appropriately and can be used for further work.

Figure 7.Isolation and Purification of PA plasmids.PA plasmids were isolated and prepped using the Giga prep kit from Qiagen. Once isolated plasmids were confirmed by DNA digestion. Lane 1 is the HindIII lambda marker. Lane 3 is NotI digested pCMVER/PA. Lane 2 is undigested. Lane 5 is KpnI digested pSecTagPA. Lane 4 is undigested.

2. Expression of PA plasmids in vitro

PA plasmids were transfected into B16 F10 mouse melanoma cells *in vitro* to confirm that PA was being expressed by the plasmids. B16 F10 cells were selected because our lab had generated standard protocols for transfecting this type of cells. Since the goal of this experiment was to confirm that the plasmids expressed PA, the cell type used was not critical as long as they were susceptible to transfection and were not killed by expression of the protein. Cells were transfected and supernatants and lysates collected after 48 hours. The results in figure 8 demonstrate that the secreting plasmid has more PA expressed in the supernatant as compared to the endoplasmic reticulum targeted plasmid. While there is more PA expressed from the ER plasmid, most of the protein is found within the lysate. The relatively even expression of PA between the lysate and supernatant in the secreted plasmids reflects a two fold increase in secretion as compared to the ER plasmid. Total expression is about the same between the two plasmids with the secreted plasmid producing about 10000pg/ml and the ER plasmid producing about 11500pg/ml.

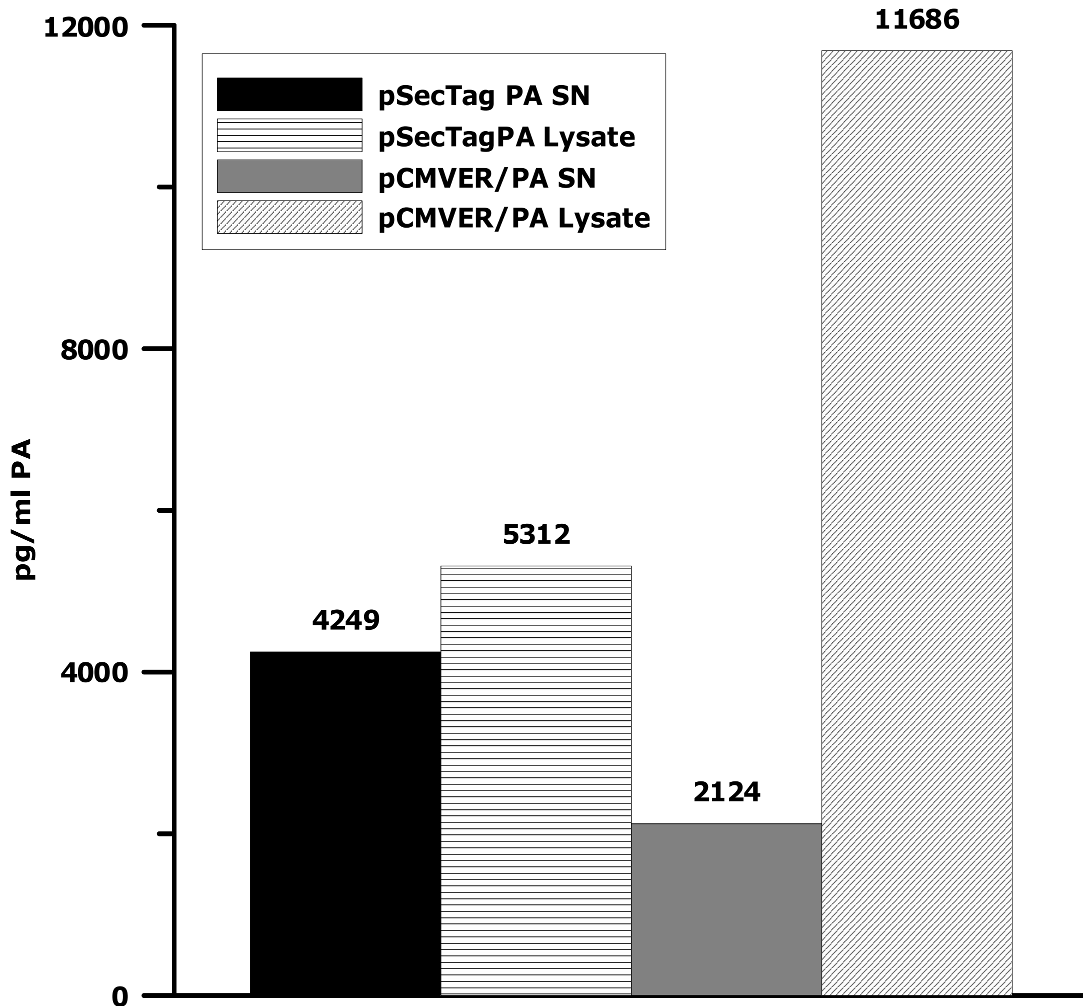


Figure 8. *In vitro* expression of PA plasmids in B16 F10 cells. Both PA plasmids were transfected into B16 F10 cells and supernatant and lysates were collected after 72 hours. Sandwich ELISA was performed to quantitate expression of PA. rPA was used as a standard for quantitation.

3. PA plasmids *in vivo*.

PA plasmids were injected into the left flank of Balb/c mice and electroporated with the MEA at 225V/cm. Each plasmid was injected individually and at a 1:1 combination to determine the amount of antibody produced by each and together. Shown in figure 9, the highest level of antibody production was seen with vaccination with the

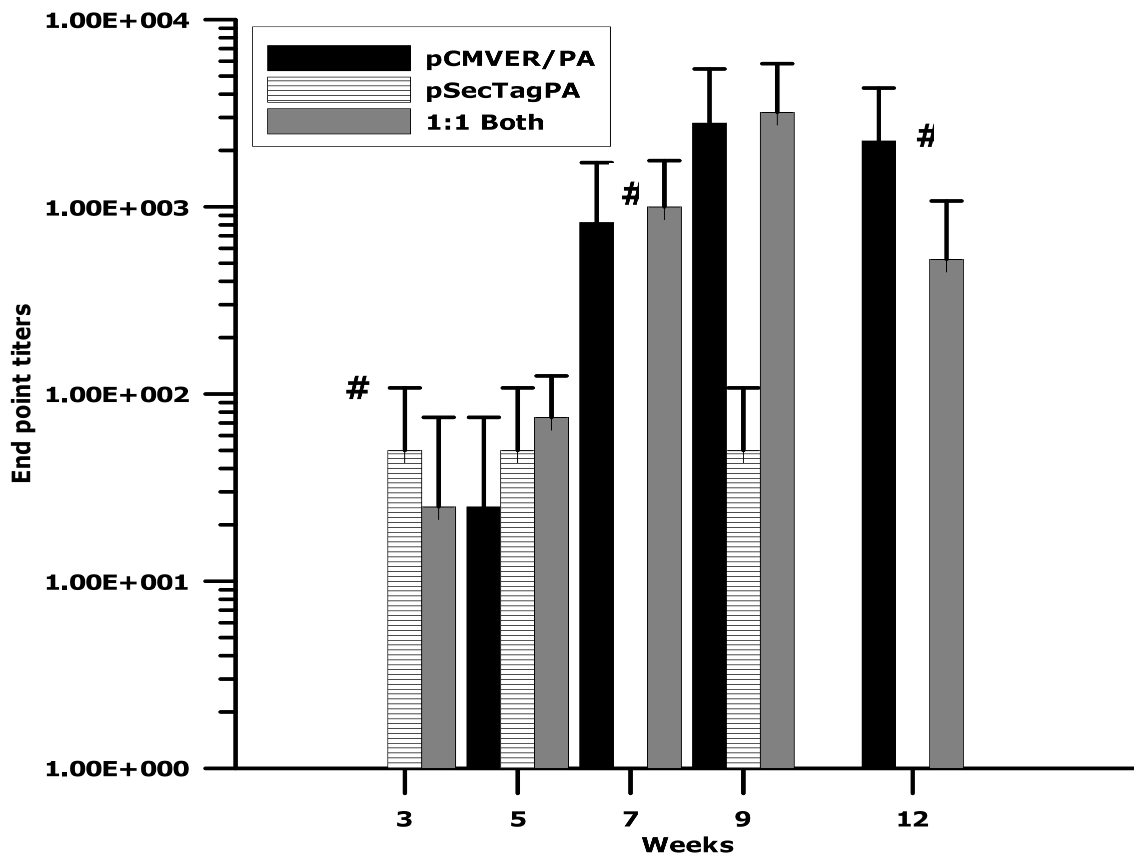


Figure 9. *In vivo* Injection of PA plasmids for the development of anti-PA antibodies. PA plasmids were injected into the left flank as described in Methods and EP at 225V/cm with either PA plasmids individually or at a 1:1 combination. **#=** value is zero

pCMVER/PA plasmid; however, antibodies were not seen until 6 weeks after initial vaccination. Vaccination with pSecTagPA produced antibodies as early as 3 weeks after treatment. Whereas vaccination with the 1:1 combination resulted in both an early response and demonstrated similar (even slightly increased) antibody levels as compared to pCMVER/PA. Based on these results, the 1:1 combination was selected for use in all future experiments.

EP with the MEA was compared to other EP devices to determine the effectiveness of antibody production with this electrode. The 4PE was used as an alternative skin electrode for comparison of skin EP and the 4 needle was used to facilitate comparison to muscle EP. DNA was injected into the left flank for skin EP as described in methods. Muscle groups were injected into the gastrocnemius and the electrode inserted into the muscle around the injection site and EP administered. Figure 10, shows that at early timepoints IO animals have higher expression than muscle injected groups demonstrating the benefit of using skin as the delivery location. Also at week 3 EP, regardless of electrode type, increases antibody production as compared to IO but are not different from each other. MEA EP samples increase steadily over time whereas muscle EP animals peak by week 9 and begin to drop off by week 12. Additionally, when comparing MEA skin delivered groups to 4PE skin delivered groups there is a noticeable difference in

antibody production. It is important to note, that even with lower levels of expression, Figure 3, the MEA can produce equal or slightly greater antibody production compared to the 4PE.

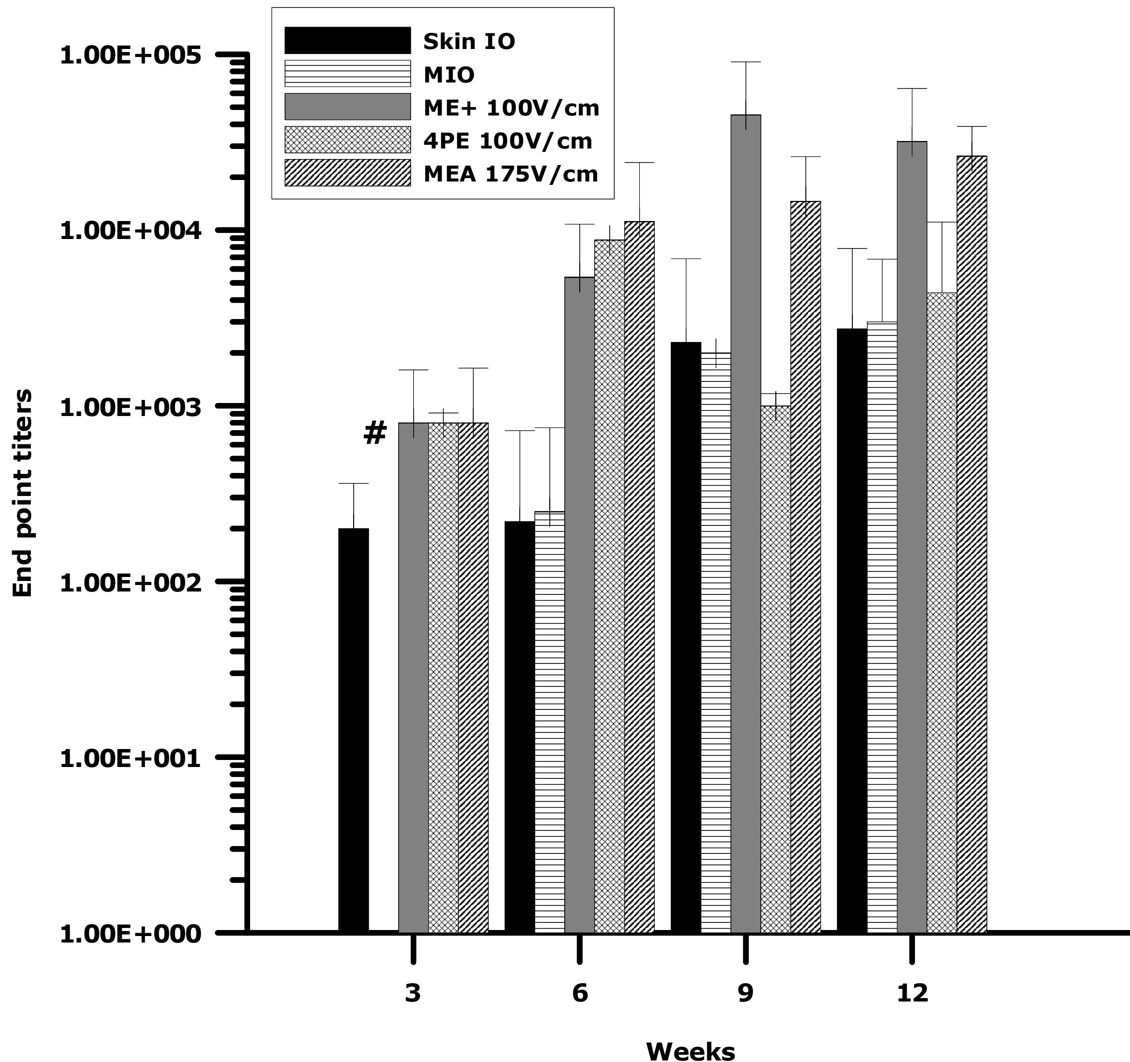


Figure 10. Comparison of MEA mediated in vivo delivery with other EP devices for antibody production. PA plasmids were combined 1:1 and injected into Balb/c mice. MEA and 4PE delivered plasmids were injected i.d., whereas 4 needle delivered plasmid was injected into the gastrocnemius. Serum was collected over time by tail vein bleed. IO= Injection only; MIO= Muscle Injection only; ME+ 100V/cm= Muscle + Electroporation at 100V/cm; 4PE 100V/cm= four plate electrode at 100V/cm; MEA 175V/cm= Multielectrode array at 175V/cm. #= value is zero.

Sub Aim b: Optimization of delivery parameters for DNA vaccination with the MEA against Bacillus anthracis

Introduction

Current experiments testing for vaccines against PA include recombinant protein, spore, and DNA constructs. Most of these vaccines are derived from the PA protein of *B. anthracis* because antibodies against this protein have been shown to have neutralizing toxin properties. However, several groups are also evaluating spore proteins either by DNA delivery, recombinant protein or inactivated whole spores. These vaccines have been shown to have some efficacy but are not capable of generating responses against toxin components. The DNA based vaccines, have tested both toxin and spore components. Two groups have tested the efficacy of muscle EP delivery of PA DNA. Their results demonstrated that this method can be used to generate total and neutralizing antibodies. As previously stated our goal is to use non-invasive EP, namely the MEA, to generate these responses. However, the EP and DNA delivery conditions cannot be assumed to be the same as in muscle delivery. Here we assess the appropriate delivery conditions for i.d. DNA vaccination against *B. anthracis* with the MEA. The three parameters tested were: plasmid dose, number of treatments, and electric field.

1. Identification of plasmid dose necessary for optimal DNA vaccination against *B. anthracis*.

Plasmid DNA was injected into the left flank of Balb/c mice in various amounts from 100-300ug. EP with the MEA at 225V/cm was used for all plasmid doses. IO was delivered at 200ug. Antibody responses were measured by ELISA. The results, Figure 11, show very little differences in antibody production. There was no detectable response from IO animals at weeks 3 and 6. All conditions are increased above IO at all time points. However, at early timepoints 200µg seems to be slightly increased over 100µg and 300µg. By week 9, 300µg had surpassed 200µg and maintained at week 12. These results do not generate any significant benefit to increasing plasmid dose. The largest differences affected by plasmid dose occurred at weeks 3-6. Over this time 200µg of plasmid shows the largest differences between groups. For this reason, we have selected to continue further experimentation with that dose.

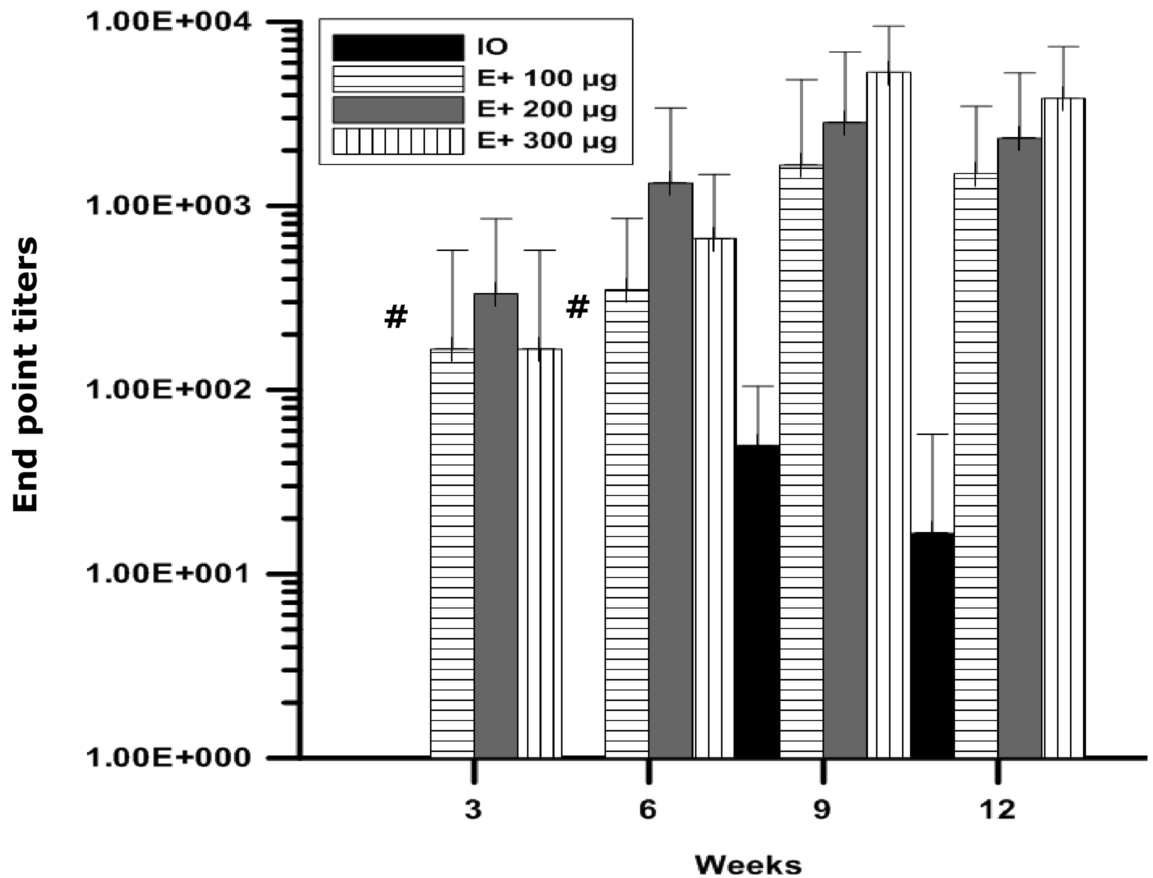


Figure 11. Effect of Plasmid dosing on generation of antibodies from MEA mediated delivery. Plasmid DNA ranging from 100 to 300ug was injected into the flank of Balb/c mice and EP with the MEA at 225V/cm. Serum was collected over time by tail vein bleed. #= value is zero.

2. Identification of the number of treatments necessary for optimal DNA vaccination against *B. anthracis*.

Animals were treated on either Day 0 and 14 or Day 0, 14, and 28. Antibodies were measured and plotted over time. The results, Figure 12, again show very little differences in antibody production. All groups increase over time and are increased over IO. However, by week 12 the group receiving the third treatment has begun to drop off, whereas the two treatment group is still increasing. This data demonstrates that there is not an additional benefit gained from a third treatment. All further experiments were conducted with a two treatment protocol at Days 0 and 14.

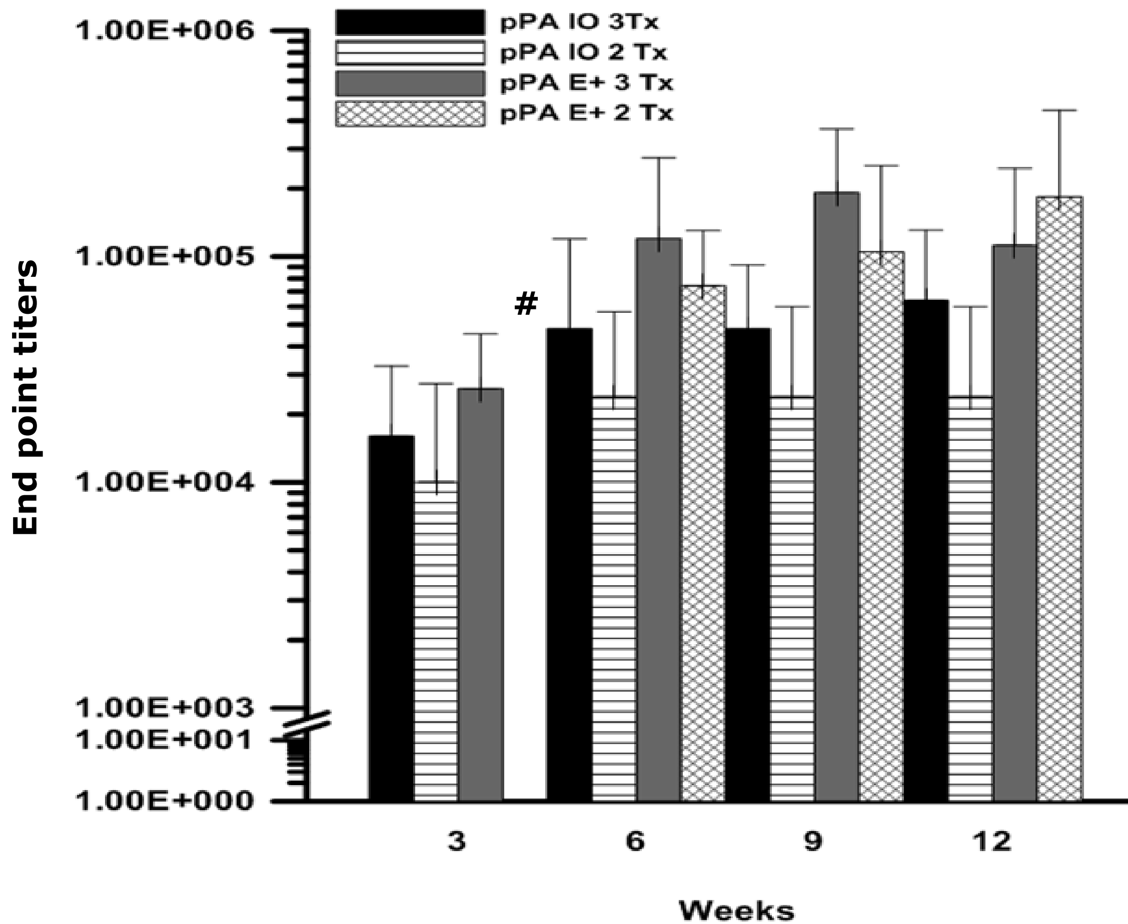


Figure 12. Evaluation of number of treatments for generating MEA mediated antibody responses. Plasmids were injected at Day 0 and 14 or Day 0, 14, and 28. Each treatment was immediately followed by EP at 175V/cm. Serum was collected over time by tail vein bleed. #= value is zero.

3. Identification of the electric field necessary for optimal DNA vaccination against *B. anthracis*.

Mice were injected with PA plasmid DNA and followed immediately with EP at electric fields from 25 to 175V/cm. Serum was collected and antibodies measured over time. Results, Figure 13, show that electric field does significantly affect antibody production. Statistical significance was determined using ANOVA with the Tukey-Kramer multiple comparisons test. Electric fields below 125V/cm generate significantly less antibodies than 125 (weeks 9 and 12) and 175V/cm (week 12). Both 125 and 175V/cm are significantly increased over IO at weeks 9 and 12. While not significantly different from each other, 125V/cm does induce slightly higher antibody responses than 175V/cm at all time points from 3-12 weeks.

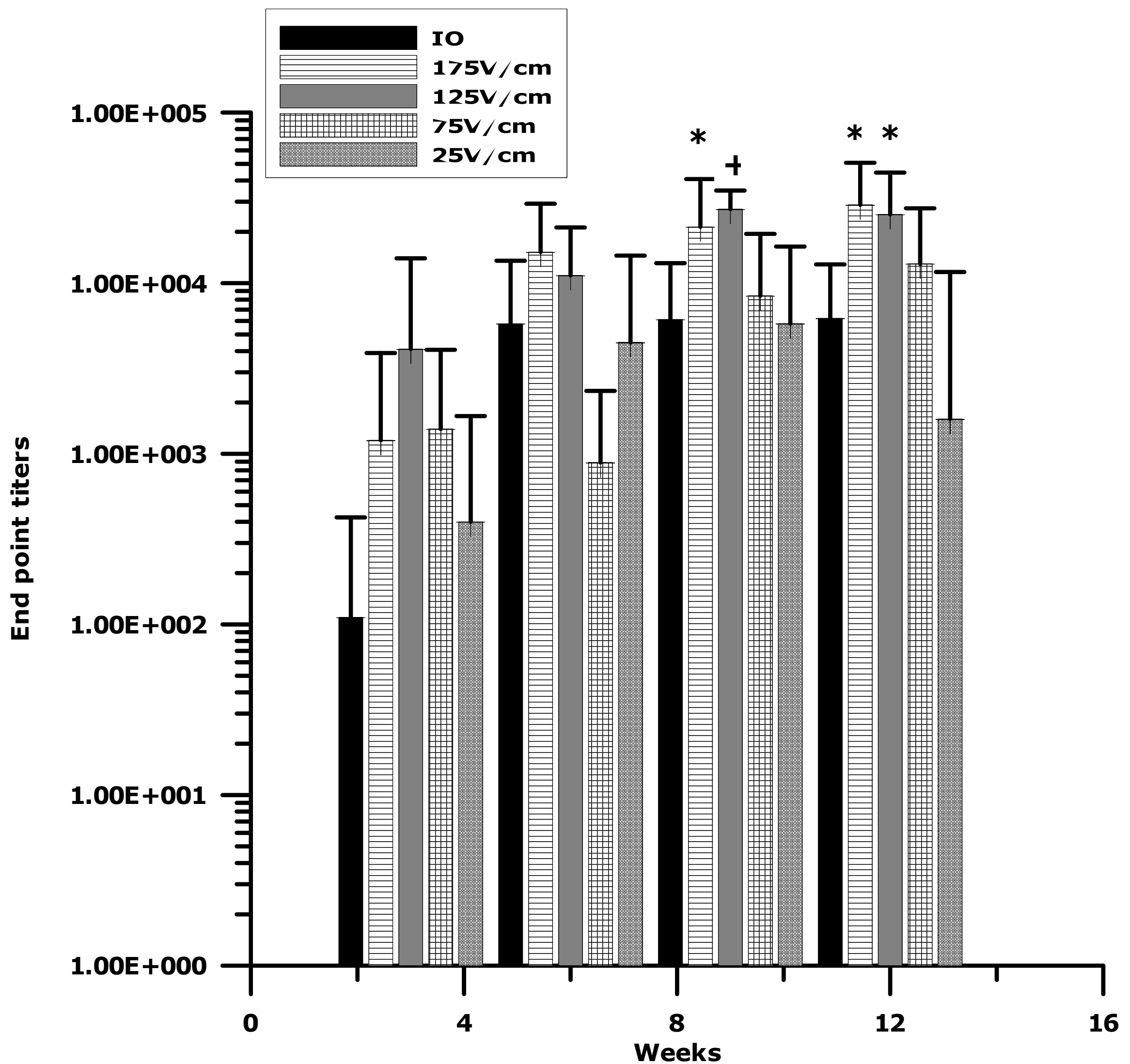


Figure 13. Evaluation of electric field from MEA mediated EP of PA plasmids for the generation of antibodies. PA plasmids were injected into Balb/b/c mice and EP with the MEA at fields ranging from 25 to 175V/cm. Serum was collected over time by tail vein bleed. Statistical analysis was performed using ANOVA and the Tukey-Kramer multiple comparisons test. *=p<0.05 compared to IO; += p<0.05 compared to all lower EP

Sub aim c: Vaccine potential of the MEA against *B. anthracis*

The critical question is whether a protective immune response can be generated using this delivery method. To assess this, a toxin neutralization assay was performed to determine the titer of neutralizing antibodies generated from our “optimized” delivery conditions. MEA EP conditions for both 125 and 175V/cm were tested as both conditions generated significantly increased antibody responses as compared to lower conditions and IO (Figure13). Table 5 shows that 3 out of 5 mice could generate neutralizing antibodies using the MEA at 175V/cm and 2 out of 5 for 125V/cm. IO and EP only groups did not have any neutralizing activity.

Table 5. *In vitro* protection by Toxin Neutralization Assay.

Condition	Peak serum titer	Serum Dilution	# of mice with TNA's
pPA IO	3200	50	0 of 5
Backbone + EP 175V/cm	0	50	0 of 5
pPA + EP 175V/cm	12800	50	3 of 5
pPA +EP 125V/cm	25600	50	2 of 5
rPA 10μg i.m.	50000	50	5 of 5

*Serum was diluted 1:50 and combined with PA to prevent toxin formation. Peak serum titers are expressed as total average end point titers for each condition.

Aim 3: Evaluate the potential for translation of electrically mediated delivery with the MEA in a human-like skin model.[1]

Introduction

This is the beginning text of the section containing previously published information [97]. Utilizing the *B. anthracis* model allowed us to evaluate this approach for DNA vaccine delivery in a clinically relevant infectious disease model, it also needs to be understood that the approach and particularly the electrode array had not as yet been tested in humans. Therefore, it was also important for us to evaluate the development of immunity and assess the condition of the skin from treatment with the MEA in a human like skin model. The best ***small*** animal model for human skin is the guinea pig. Their skin is approximately the same thickness (about 1mm) and contains similar properties for antigen presenting cells [63]. Hairless guinea pigs are the best model because while they still have hair follicles they do not possess the fur that normal guinea pigs do, however due to an infection in the hairless guinea pig population it was not feasible to use

[1] Portions of these results have been previously published (Donate, A et al 2011 [97]) and are utilized without need for publisher permissions due to the Creative Commons License. Legal Code is included in Supplementary Materials. Level of work contributed by Authors: Amy Donate 70%, Yolmari Cruz 5%, Domenico Coppola M.D. 5%, and Richard Heller Ph.D. 20%.

this model for vaccination testing. Therefore, we used regular guinea pigs and their hair was shaved.

Our initial experiments with this guinea pig model were to evaluate expression at various EP conditions with the MEA and to compare those to our current 4PE skin electrode. Our results published in Human Gene Therapy in 2010 demonstrated that the MEA was an efficient delivery electrode for gene expression in the guinea pig. Luciferase expression equivalent to the 4PE could be achieved as low as 250 to 300V/cm and 150ms. Additionally, we could increase gene expression by increasing the area of skin EP [65]. MEA EP with GFP plasmid showed that this expression was localized to the epidermal and dermal layers of the skin. These results make the MEA a good candidate for vaccination in our human like skin model.

a. Plasmid expression from EP.

The first step in evaluating the MEA for delivery of DNA vaccines in a human-like model was to evaluate expression of gWiz™ HBsAg, Figure 14. Guinea pigs

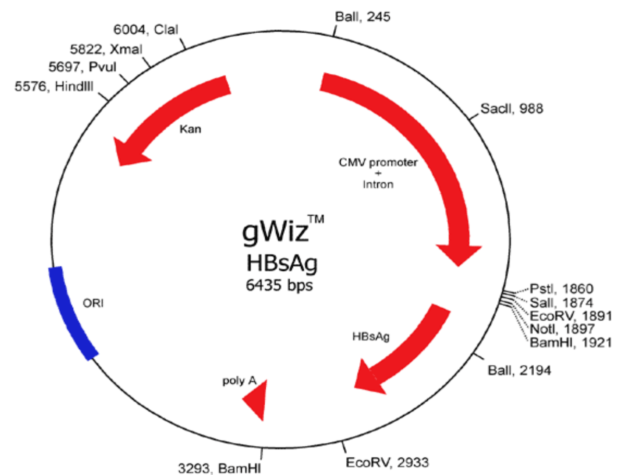


Figure 14. Gwiz™HBsAg plasmid. Map image from aldevron.com

were treated as described in Methods with or without EP using the MEA at 300V/cm. Guinea pigs were humanely euthanized 48 hours after delivery and the treated skin harvested and snap frozen. Expression of HBsAg was determined by immunohistochemistry.

In Figure 15 A and B, expression of HBsAg is seen in IO and MEA EP samples. Increased staining compared to IO samples can be observed in the MEA EP samples. Expression of HBsAg is seen within the epidermis of both groups, but is in much higher quantity in the MEA EP group. Additionally, deeper expression into the dermis is also noted in the MEA EP condition. It should also be noted that there is a slight separation of the epidermis in the MEA EP group. This damage was evaluated in further experimentation and found to be minimal and completely recoverable over time, Figure 16.

b. Immune cell infiltrate and tissue damage

Other than expression of plasmid DNA, another important factor for developing immunity is the recruitment of immune cell infiltrate. This is an important consideration especially for DNA vaccination because it can often be a limiting factor for this type of vaccine. To test for this, skin sections were collected 96 hours after treatment, frozen, and stained with H & E. Induction of immune cell infiltrate was observed (Fig 15 C-F 100X magnification). Background levels, Fig 15C, of infiltrate are demonstrated in no treatment control and

correspond to low levels of cellular infiltrate (purple). IO samples show slight increases in infiltrate as compared to no treatment, Fig 15D. In contrast, MEA EP samples show a large increase in cellular infiltrate, Fig 15E. The substantial influx of immune cells can be seen more clearly in figure 15F (200x magnification MEA EP).

It is important to observe that edema was noted in all injected tissues. This is most likely a result of the injection and not of the EP as it is seen in the IO samples as well. Edema did not appear increased due to EP. Additionally, in most samples, tissue damage and necrosis were not seen. However, two EP delivered samples had minimal ulcerations at 96 hours after treatment, one of which also had about 1% necrosis. There were no other samples showing damage or necrosis. This is further evaluated by macroscopic evaluation of the skin. Injection site redness is seen immediately after treatment, with a slight increase in redness in the MEA EP treated sites, Figure 16. By 48 hours after treatment most of the redness has cleared up and IO and MEA EP groups are indistinguishable from one another. Complete visual recovery of the skin is seen by Day 7.

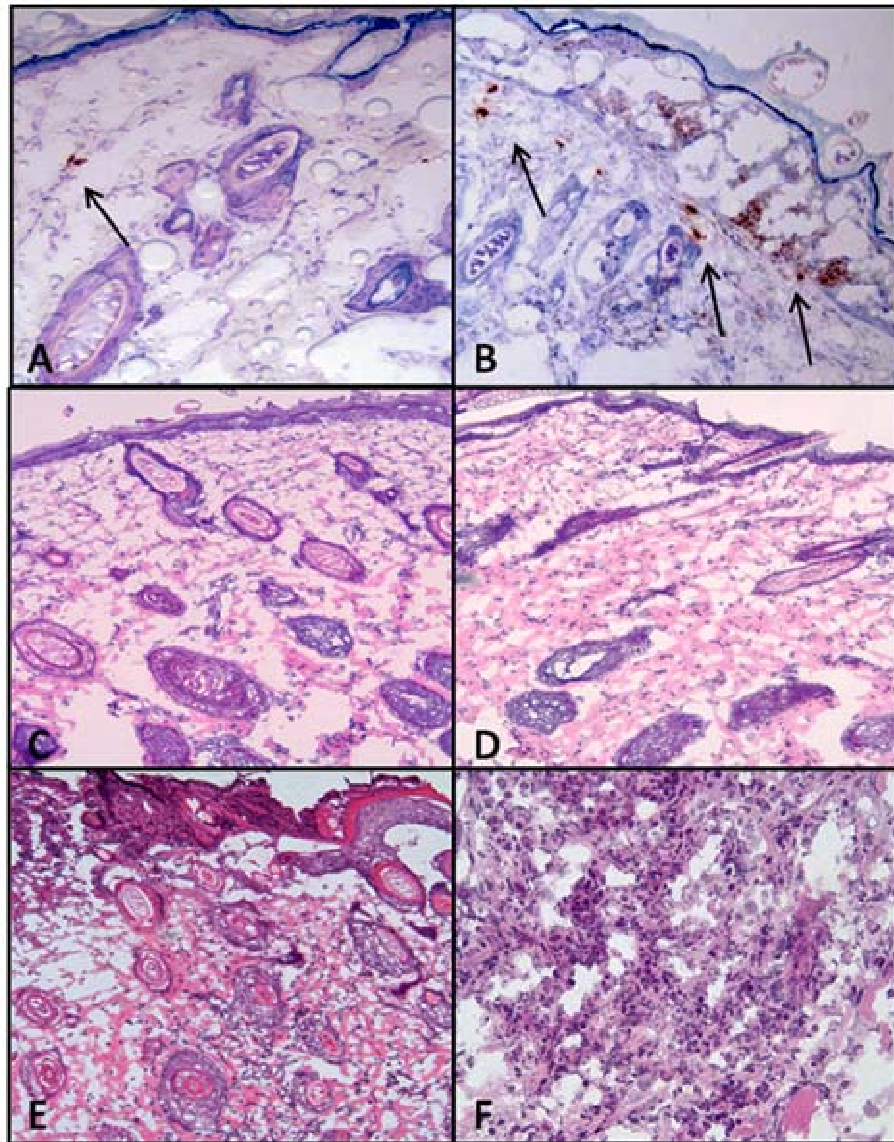


Figure 15. Histological Assessment of Guinea pig Skin for Expression, Inflammation, and Damage from MEA Mediated EP. Female guinea pigs were injected on the left flank with pGwizHBsAg. Immunohistochemistry was performed to determine expression of HBsAg after 48 hours A) IO and B) EP 300V/cm. Skin samples were collected for H & E staining 96 hours after treatment with the MEA at 300V/cm. C) No tx, D) IO, E) EP 300V/cm F) EP 300V/cm 200X magnification.

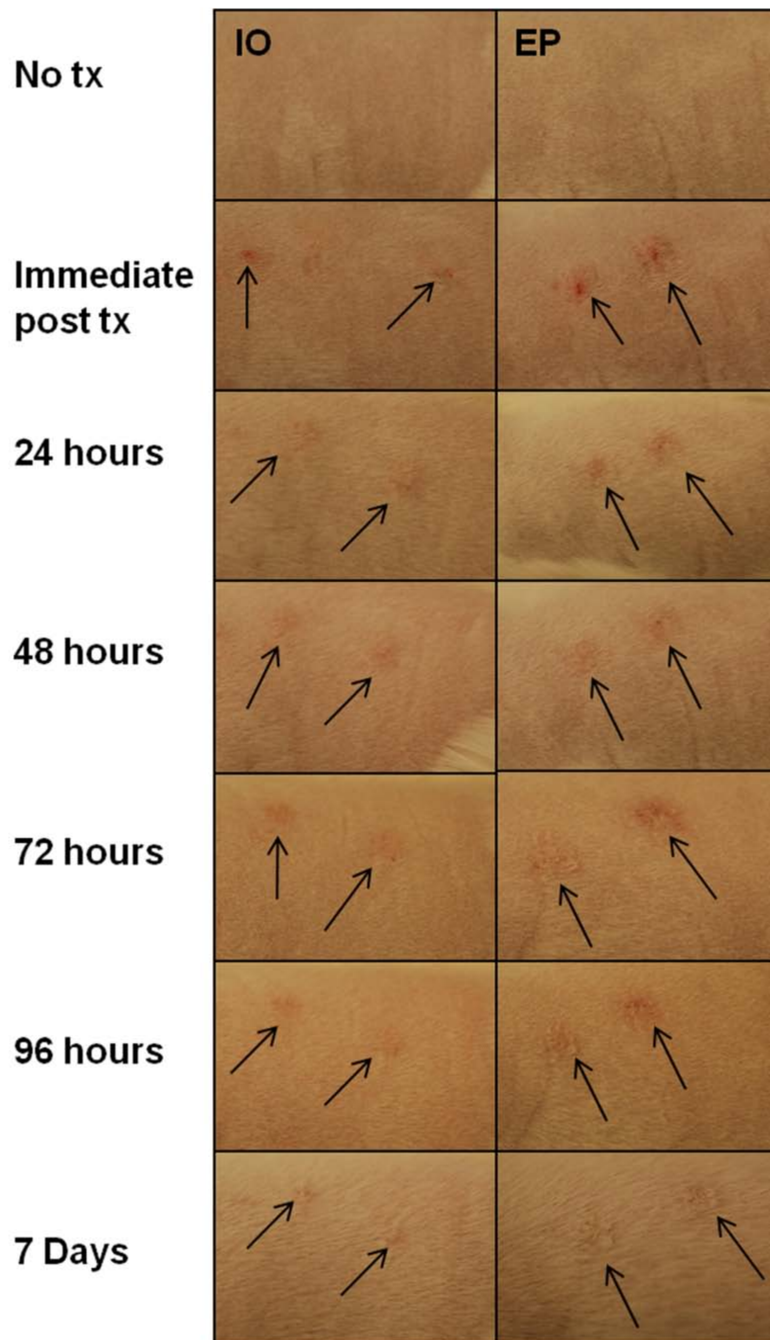


Figure 16. Visual assessment of guinea pig skin for damage and recovery. Guinea pig skin was imaged immediately after treatment through Day 7 to determine damage and healing.

c. Anti-Hepatitis B surface antigen antibodies

The development of specific immunity is a more accurate indicator of an effective immune response from treatment. Therefore, serum was collected from treated guinea pigs and anti-HBs were measured by ELISA over time. Results, Figure 17, show significant increases in antibody expression by week 3 and those responses

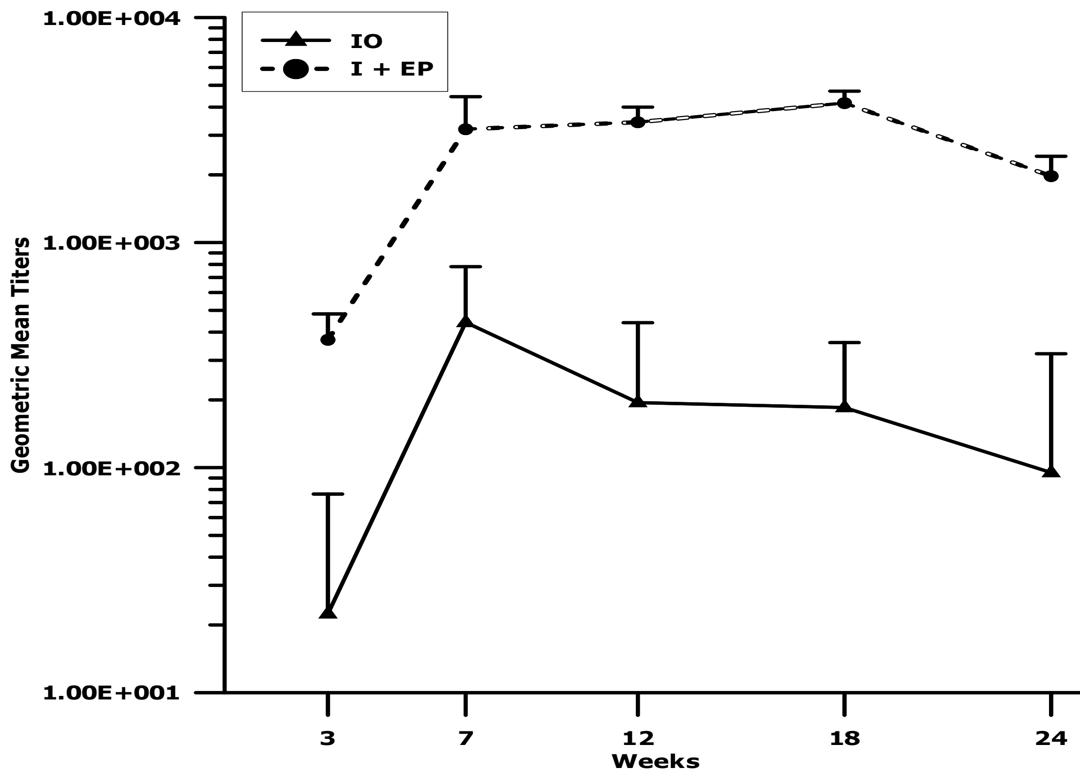


Figure 17. Evaluation of the development of humoral immunity from MEA mediate EP in a human like skin model. Guinea pigs were injected on the left flank and EP groups were immediately EP with the MEA at 300V/cm. Guinea pigs were bled by jugular vein over time and serum used for ELISA. Both groups had an n-=18 from three independent experiments. Statistical analysis was performed using a student t-test and bonferroni correction.

remain significantly increased through week 24. The results are represented as GMT with IO groups having a peak GMT of 1000 and MEA EP at 5000 (Figure 17). The peak fold increase of MEA EP over IO was 6.5 at week 18. However, the fold increase remained relatively constant at about 5 for all time points. The titers measured do correlate with titers conferring protection [22, 41, 51, 119, 120]. Statistical analysis was performed using student t-test and bonferroni correction to correct for multiple timepoints. Error is represented as standard error of the means.

Discussion

The data presented in this dissertation reflect that the MEA can be an effective mediator of gene expression, inflammation, and humoral immunity and that those responses are highly dependent on the electric field used in both mice and guinea pigs. DNA vaccination is advantageous because it does not integrate into the host DNA, it is cost effective to produce and easily stored, it can be highly specific for tissue and/or cell type and can be made to vaccinate against multiple agents simultaneously. The skin is an ideal target for DNA vaccination due to the large surface area and presence of antigen presenting cells like langerhan's and dermal dendritic cells, specialized for induction of immunity [98].

However, injection of plasmid alone does not induce high enough immune responses to be protective. EP is one method that has been shown to increase both plasmid expression as well as immunity. Previous EP methods have involved painful penetrating electrodes that go into the muscle to facilitate delivery. Further advancements have been made using non-penetrating electrodes such as caliper and plate

electrodes. However, these electrodes require high voltages to enhance delivery and therefore can cause tissue damage.

Our initial experiments in mice evaluated the gene expression from intradermal delivery with either the MEA at various field strengths or the optimized 4PE at 100Vcm. The data (Figure 3) showed that while gene expression with the 4PE represented the highest expression of luciferase the MEA could increase luciferase expression above IO and that by increasing the electric field could be made similar to the 4PE in mice. MEA EP conditions above 200V/cm were originally tested but resulted in visual damage of the skin. The goal of this dissertation was to determine the effectiveness of this method for use in DNA vaccination; therefore, visual tissue damage is not considered an acceptable side effect. Therefore only those conditions resulting in no visual damage were used in the rest of the studies (conditions of 175V/cm or less). Previously published results in rats demonstrated that at higher electric fields of 250V/cm the MEA could actually result in higher gene expression than the 4PE [21] and this was later also demonstrated in guinea pigs [55]. The differences in gene expression with the MEA and 4PE between these animal models is most likely related to skin thickness and structure of the two larger rodent models. Both of these animals have thicker skin than mice and

therefore can be more easily injected into the dermis as well as having a more substantial network of cells for expression.

Upon evaluating the effect of the MEA on damage and inflammation in mice, we were able to determine that EP with the MEA did induce inflammation and cellular recruitment when injected with plasmid DNA. Edema was also seen but it was not isolated to EP delivered animals and is most likely an injection effect. The cellular infiltrate seen at 48 hours after treatment is most likely not from a specific response to antigen. Rather it is an effect from MEA EP. This is a benefit to using EP as the delivery method for DNA vaccination. It has previously been shown, and this data supports the idea that EP has an adjuvant effect.

While overall gene expression is an important factor for determining electrodes and electric fields another important consideration is where the plasmid DNA is being expressed. Are there a large number of cells with a low copy number of plasmid or are there a few cells with high copy numbers? We proposed to answer this question by injecting with GFP and looking at GFP expression at 24 and 48 hours after treatment (Figure 5). IO was used as a control and we evaluated high and low electric fields, based on luciferase expression, using the MEA. We were able to determine that expression was low at 24 hours but could be seen in the EP samples

(both 125 and 175V/cm). However, at 48 hours GFP expression can be seen in the IO samples. MEA EP with 125v/cm appears to still have higher expression and that expression is seen throughout the epidermis and to some extent in the dermis. MEA EP with 175V/cm showed fewer cells expressing GFP than either IO or MEA EP at 125V/cm and that most of the expression was within the dermis. Based on the luciferase expression in Figure 3, we show that the total expression with MEA EP at 175V/cm is approximately two fold higher than with 150V/cm. It would stand to reason, given the trend of increasing gene expression with increasing electric field, that 125V/cm would demonstrate even lower expression levels than 150V/cm. The difference between these groups may be the number of cells transfected, Figure 5. When using higher electric fields more cells are killed during treatment and therefore less cells can be transfected, but those cells that survive are more greatly "porated" and can allow a greater amount of DNA into the surviving cells. In the case of the lower electric field, less DNA can be taken up into the cells but more cells are moderately porated. This results in a wider spread of DNA uptake and expression. Increases in cellular infiltrate may also represent protective effects that are not represented by measurement of antibody responses and neutralizing activity. Early work conducted with the gene gun was also suggestive of this effect [99, 100]. In that

study, toxin neutralization was quite low and in several cases non-existent; however, several of these animals were protected in lethal challenge assay. The question remains, which of these conditions is more important in regards to DNA vaccination? This was the goal in Aim 2; to evaluate what vaccinations conditions resulted in the highest levels of humoral immunity.

To evaluate the development of humoral immunity against a clinically relevant infectious agent we used *B. anthracis* as a model. While *B. anthracis* has a currently available vaccine it is not used for the general population due to the side effects as well as the heavy initial inoculation schedule. The goal with development of new vaccines for *B. anthracis* is to reduce the production burden, side effects, and the number of initial inoculations necessary for vaccination. It also has a well-studied small rodent model in the mice, as well as highly correlated in vitro assays of protection. We received two plasmids from Hahn et al to study the development of DNA vaccination with the MEA. These plasmids expressed the full length PA protein in commercially available backbones from Invitrogen. These backbones consisted of either a secretion sequence (pSecTag2B) or a sequence targeting the endoplasmic reticulum to generate a membrane bound form of the protein (pCMVER). Once the plasmids had been purified (Figure 7), we evaluated expression in vitro and in

vivo. Both plasmids performed as expected. In vitro, expression of PA from pCMVER/PA was found primarily in the lysate, whereas expression from pSecTagPA increased expression in supernatant about two fold. In vivo injection of both plasmids individually resulted in the development of anti-PA antibodies when delivered by EP with the MEA. Total antibody production was highest with pCMVER/PA but was not seen until week 6. Total antibody production from electrically mediated pSecTagPA was seen at early timepoints but had waned by week 9 and did not reach as high levels as pCMVER/PA. We combined the two plasmids in a 1:1 ratio (pPA) to determine if this would generate a combination of these two responses. The result was both an early and high antibody response that lasted at least 12 weeks providing the benefit of both plasmids. This information supported the stated report by Hahn et al 2004 [99].

How does EP with the MEA compare to other electrode devices for the development of humoral immunity? Muscle EP has been demonstrated to induce high levels of humoral immunity due to the long lasting expression of protein in the muscle. However, it is not naturally an immune generating system. The skin, however, is an ideal target for DNA vaccination. We evaluated our 4 needle muscle electrode, with the MEA, and the 4PE to determine how well the MEA can induce humoral immunity. Our results generated equal responses

at early timepoints regardless of electrode used. However, at week 6, antibodies from skin EP devices had increased slightly above muscle EP. EP with the 4PE began to decrease by week 9, at which time the muscle EP peaked above both skin electrodes. EP with the MEA steadily increased even through week 12 when muscle EP began to decrease. Three interesting points should be mentioned. First, differences were seen in the IO groups. Skin IO responses were seen as early as week 3, whereas muscle took until week 6 to develop. This supports the immune stimulating idea of using the skin. Second, even though when the 4PE generated higher luciferase gene expression compared to the MEA, this was not seen when evaluating antibody responses which showed that delivery with the MEA generated higher responses. Therefore, based on these results, the MEA is a superior electrode as compared to the 4PE for stimulating immune responses. Finally, EP with the MEA generates similar immune responses as muscle EP over time and may maintain that immunity over longer periods of time.

Our initial studies were very promising, so we set out to determine the optimal delivery parameters for development of humoral immunity using the MEA. The three parameters we evaluated were amount of plasmid, treatment course, and electric field. Only small differences were seen in varying the amount of plasmid, and

none of these differences were significant. However, we could see small increases from increasing the amount of plasmid, but we deemed this irrelevant as injection with 200μg was the highest at weeks 3 and 6. Similar results were seen with the treatment course, either a two treatment course at Days 0 or 14 or a three treatment course at Day 0, 14, and 28 were evaluated. While there were only small differences seen between these two treatments it did appear that the three treatment condition started to decrease antibodies earlier than the two treatments. This may be able to be explained by plasmid clearance. It was reported that too much plasmid in the skin can result in faster clearance of the plasmid [101]. If this is the case then development of humoral immunity could be even more limited by a decrease in the time of expression from over treatment. As noted in our other experiments the two treatment course appears to still be increasing at week 12. Finally, evaluating the effect of electric field on development of humoral immunity resulted in significant differences. At all timepoints MEA EP with 125V/cm demonstrated the highest levels of antibody production. This may be explained by the GFP data presented earlier in this dissertation where a larger area was expressing plasmid than in the higher EP conditions. Interestingly, 125V/cm demonstrated significantly increased responses as compared to all lower conditions at weeks 9 and 12 making it the optimal MEA EP

condition for the development of humoral immunity against B. anthracis. However, conditions as high as 175V/cm can be used and demonstrate significant increases as compared to IO and EP conditions lower than 125V/cm as well thought 125V/cm is slightly higher they are not significantly different from one another.

Additionally, when conducting our neutralizing assays, those animals that did not elicit 50% protection, and were therefore not considered protected, did still demonstrate some protective effects at 20-40%. This was not seen in the IO or backbone controls where at least 95% cell killing occurred. Despite not being enough protection to be considered "protected", there was some response in every MEA treated animal at 175 and 125V/cm.

Our results compare favorably to most other published DNA vaccines in the Balb/c model [99, 102-106]. Those studies reporter higher total IgG in Balb/c mice required additional boosters, addition of recombinant protein boosts, [107, 108] or the use of signaling adjuvants. Studies conducted in other mouse models have shown antibody responses that exceeded our results [40, 45, 100, 109, 110]. This may be explained by differences in the immunogenicity of the models. Balb/c mice are not highly susceptible to challenge from non-encapsulated toxigenic strains of *B. anthracis*, whereas mouse models like A/J mice are highly susceptible [111]. In one study, that used

both A/J and Balb/c mice evaluated the development of humoral immunity against *B. anthracis* was evaluated. The A/J mice developed almost a ten-fold higher response than the Balb/c mice [100].

Our results compare favorably to muscle EP as well. Two other studies, one in mice [45] and one in non-human primates [93], have been conducted specifically evaluating the use of EP to deliver a *B. anthracis* vaccine. Our results are similar to the mouse study demonstrating approximately 25000 titers and peaking at similar time points between 6-9 weeks. While our study required additional DNA, the use of the non-invasive MEA provides a positive advancement to this study. The NHP experiment was conducted with penetrating needle electrodes into the muscle. Their results showed the development of protective immune response [93]. While it would be difficult to compare our results to these, we feel that their data corroborate the claim that EP could be an effective delivery method for DNA vaccination against *B. anthracis*. We believe our method may be a way of making this treatment even more tolerable and reducing the invasiveness.

In aim 3 we evaluated the effect of using the MEA for DNA vaccination in a human-like skin model. EP with the MEA generated increased plasmid expression as well as an increase in immune infiltrate after treatment. The magnitude of immune infiltrate was

greater in EP groups than IO and there was minimal to no skin damage associated. Specific, lasting, and significant levels of antibodies were greater than IO. This is the first report to demonstrate the use of the MEA for DNA vaccination in a human-like skin model.

As expected from our previous publication [55], EP with the MEA enhanced expression. While the exact mechanism involved in EP remains unknown, increased plasmid expression at least in the case of DNA vaccination, plays an important role in recognition by the immune system [112]. EP has been shown to have an adjuvant effect by recruiting immune cells to the site of pulse application [2]. In our study, we saw an influx of nucleated cells from EP treated samples, shown in figure 14. These cells are most likely neutrophils and macrophages based on morphology. This is most likely a combination of both an EP mediated adjuvant effect and increased plasmid expression. The induction of macrophages and polymorpho-nucleated neutrophils is indicative of a chronic inflammatory response. While the perception of prolonged inflammation is typically negative, in our case it indicates that the expression of the plasmid is present for a prolonged period of time, giving the immune response enough time to perform its function. Based on our earlier work, we would expect this prolonged expression to decrease after approximately 14 days, therefore allowing the body to heal and not generate deleterious

effects from inflammation [1, 55]. As shown in Figure 15, we can see that any visual effects from EP inflammation have recovered by Day 7.

These findings seem to correlate with our antibody data, where an increase in the presence of specific antibodies was measured over time. These antibodies were significantly increased as compared to injection only. GMT's ranged from 4000-16000 peaking at week 18. Antibody levels remained elevated until dropping off after week 21, but still remained increased as compared to injection only. The enhanced intensity of humoral immunity by EP with the MEA corresponds to previously published skin EP results [113-116]. One of the primary reasons for evaluating our delivery method with HBV was because it is a well characterized vaccination model. Published studies have reported geometric mean titers in conjunction with protective efficacy in guinea pigs. While the presented GMT's in these papers were higher than ours, they also reported protective levels more than 100 fold above the necessary levels of 10mIU/ml. Our GMT's are likely to still be within the protective range without generating unnecessary additional responses [117, 118]. Compared specifically to HBV DNA vaccines delivered by EP several animal models have been evaluated and EP has been shown to have protective levels from 10-1000mIU/ml [22, 41, 51, 119, 120]. The most recent comparable publication evaluated a minimally invasive device for protective vaccination

against influenza [120]. While their results were only presented as neutralizing titers against flu and cannot be compared directly we believe that our electrode design generates immune responses of equal quality without tissue penetration.

Conclusions

In this study we evaluated the use of the MEA for increased gene expression, inflammation, damage and the induction of humoral immunity. These criteria were evaluated in two infectious models as well as two animal models. Our initial results in the mouse demonstrated that gene expression could be enhanced in mice with increasing electric fields with the MEA and the highest field that could be used without visual tissue damage was 175V/cm. Additionally, differences were seen in GFP expression based on the electric field applied. Lower electric fields, which correlated to lower total luciferase expression, showed a slight increase in the number of cells transfected as compared to higher electric fields (higher total luciferase expression but less total cells transfected). Inflammation was also noted in the mouse model as early as 48 hours after injection and EP.

In our mouse model against *B. anthracis* we were able to determine that plasmid dose and number of treatments played only a small role in development of humoral immunity. Of the factors we studied, the most important was the electric field. Electric fields ranging from 125-175V/cm showed significant increases in humoral

immunity as compared to lower and IO conditions. They also correlated to 40-60% protection in our in vitro toxin neutralization assay.

Finally, in our guinea pig skin model against HBV we demonstrated that the MEA would effectively mediate increases in humoral immunity in the human like skin model. Our results showed increased gene expression, inflammation, and significant increases in humoral immunity at all time-points through 6 months as compared to IO. Slight redness was seen after treatment with the injection and MEA EP but was not greater than IO after 24 hours and was completely recovered by Day 7.

In conclusion, using the MEA for EP delivery effectively increases gene expression, immune cell infiltrates and humoral immunity in both mice and the human like skin model, guinea pig and therefore should continued to be utilized for DNA vaccine studies.

Future Directions

The MEA should continue to be evaluated for use in DNA vaccination against other clinically relevant infections. There are several areas that could be studied to more completely evaluate the effect of MEA mediated EP. First, we evaluated the induction of CMI since EP has been shown to enhance this type of immunity. Based on our results showing cellular infiltrate the MEA is likely having an effect on cell mediate immunity as well. Specifically, induction of CD4+ and CD8+ T cells should be evaluated.

Secondly, the addition of adjuvants could enhance the immune response and may provide greater longer lasting protection. Recently, plasmid based cytokine adjuvants like IL-15, IL-2, and IL-12 have been used to enhance immunity. The appropriate adjuvant would be dependent on the infectious model, whether humoral or cell mediated immunity was important to adjuvant. Alternatively, the use of cell specific promoters could isolate DNA uptake. Our studied utilized the MEA to the skin to theoretically increase DNA uptake by antigen presenting cells, but using a promoter that would isolate DNA uptake and gene expression to APC's or more specifically dendritic cells for

example would increase antigen presentation and may increase immunogenicity.

Finally, a benefit of DNA vaccination is that the DNA can be made to encode for as many or as few components as necessary. In the case of development of a MEA mediated DNA vaccine against *B. anthracis* plasmid addition of spore components with the toxin component may provide greater protection during an actually inhalational infection by providing immunity long before toxin production. Alternatively, more highly optimized plasmids expressing smaller portions of PA (namely the binding epitope domain IV of PA). Using only very specific regions of PA would eliminate excess antibody production to generate only those antibodies that would inhibit toxin formation and enhance protection.

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Appendices

Appendix A: Publications and Posters by Author

Primary Articles

Donate, A., Heller, R. Non-invasive electrically mediate DNA Vaccine against B. anthracis. Submitted Oct. 2011, Molecular Therapy.

Donate A., Coppola D., Cruz Y., Heller R. Evaluation of a novel non-penetrating electrode for use in DNA vaccination. PLoS One. 2011 Apr 29;6(4):e19181

Guo S., **Donate A**., Basu G., Lundberg C., Heller L., Heller R.(2011). Electro-gene transfer to skin using a non-invasive multi-electrode array. J Control Release.2011 May 10;151(3):256-62. Epub 2011 Jan 22

Ferraro B., Heller L., Cruz Y., Guo S., **Donate A**., Heller R. (2010) Evaluation of delivery conditions for cutaneous plasmid electrotransfer using a multi-electrode array. Gene Therapy 2011 May;18(5):496-500. Epub 2010 Dec 23

Poster Presentations

Donate, A.; Heller, R. Electrically Induced DNA Vaccine Against *Bacillus anthracis*. American Society for Microbiology General Meeting May 2011.

Donate, A.; Heller, R. The Multi-Electrode Array: Evaluation of a Non-Penetrating Electrode for Use in DNA Vaccination Against *Bacillus anthracis.* Gordon Research Conference on Bioelectrochemistry 2010

Donate, A.; Heller, R. Optimization of the multi-electrode array in a mouse model for use in DNA vaccination against *Bacillus anthracis*. International Consortium for Bioelectrics Annual Symposium 2010

Donate, A.; Heller, R. Non-invasive electrically induced DNA vaccine against *Bacillus anthracis*. American Society for Microbiology: Biodefense 2010

Donate, A.; Heller, R. Evaluation of the multi-electrode array for use in DNA vaccination against *Bacillus anthracis*. American Society for Gene Therapy 13th Annual Meeting 2010

Donate, A.; Cruz, Y.; Coppola, D.; Heller, R. Evaluation of the conformable array for use in DNA vaccination. Gordon Research Conference on Bioelectrochemistry 2008

Donate, A.; Cruz, Y.; Heller, R. Evaluation of cutaneous administration of DNA vaccines delivered by electroporation. Florida Center of excellence 1st annual symposium 2007

Oral Presentations

Donate, A.; Heller, R. The Multi-Electrode Array: Evaluation of a Non-Penetrating Electrode for Use in DNA Vaccination Against *Bacillus anthracis.* Gordon- Kennan Research Symposium 2010

Donate, A; Cruz, Y.; Coppola, D.; Heller, R. Evaluation of Cutaneous Administration of DNA vaccines delivered by electroporation. American Society for Gene Therapy 11th Annual Meeting 2008

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Appendix C: Published First Author Publication [97]

Evaluation of a novel non-penetrating electrode for use in DNA vaccination. (2011). *PLoS One* 6: e19181.

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Abstract

Current progress in the development of vaccines has decreased the incidence of fatal and non-fatal infections and increased longevity. However, new technologies need to be developed to combat an emerging generation of infectious diseases. DNA vaccination has been demonstrated to have great potential for use with a wide variety of diseases. Alone, this technology does not generate a significant immune response for vaccination, but combined with delivery by electroporation (EP), can enhance plasmid expression and immunity. Most EP systems, while effective, can be invasive and painful making them less desirable for use in vaccination. Our lab recently developed a non-invasive electrode known as the multi-electrode array (MEA), which lies flat on the surface of the skin without penetrating the tissue. In this study we evaluated the MEA for its use in DNA vaccination using Hepatitis B virus as the infectious model. We utilized the guinea pig model because their skin is similar in thickness and morphology to humans. The plasmid encoding Hepatitis B surface antigen (HBsAg) was delivered intradermally with the MEA to guinea pig skin. The results show increased protein expression resulting from plasmid delivery using the MEA as compared to injection alone. Within 48

hours of treatment, there was an influx of cellular infiltrate in experimental groups. Humoral responses were also increased significantly in both duration and intensity as compared to injection only groups. While this electrode requires further study, our results suggest that the MEA has potential for use in electrically mediated intradermal DNA vaccination.

Introduction

The development of vaccines is widely considered to be one of the most important medical advancements of the 20th century. Current methods have been pushed to the limits of their potential. New techniques need to be developed and employed to combat a new generation of diseases and infections. There are several advantages to DNA vaccination. DNA vaccines are cost effective to produce, they can be easily stored, they are highly specific and their multivalent nature means that they could be combined to vaccinate against several different components simultaneously [1-3]. Either due to low expression or lack of immune recognition, injection of plasmid DNA alone does not elicit a strong enough immune response for protective vaccination. Electroporation (EP) is a non viral plasmid DNA delivery approach that effectively enhances plasmid expression [4, 5] and immunity [6-10].

EP requires the application of electric fields causing permeabilization of the cell membranes. The permeabilized membrane briefly contains "pores" that allow large molecules, like DNA, to enter the cell. Initial studies evaluating *in vivo* EP for transgene delivery and

expression were performed on rat brain tumors [5] and rat livers [4]. Those studies demonstrated enhanced delivery and expression of plasmid DNA from EP mediated delivery. Successful EP mediated DNA delivery has been demonstrated in most tissue types and for several therapeutic and prophylactic indications such as cancer therapy, infectious diseases, wound healing, metabolic disorders and vaccines [11]. Recently several clinical trials have been initiated. Two clinical trials have been completed using EP, one assessing tolerability of intramuscular delivery [12, 13] and the other assessing toxicity and clinical utility of delivering pIL-12 intratumorally by EP to melanoma patients [14]. The latter demonstrated the safety, minimal toxicity, and feasibility for the use of EP in the clinic [14]. Since the successful completion of these studies, 19 others are currently active or recruiting. Five of those are involving DNA vaccination against infectious agents (clinicaltrials.gov; Keyword: Electroporation).

Initial *in vivo* EP DNA vaccine studies evaluated gene expression and immune stimulation from delivery of plasmids encoding either Hepatitis B Virus (HBV) protein or Human Immunodeficiency Virus (HIV) protein, gag, to the muscle. Their results confirmed that increased humoral responses to HBV [6] and cellular [9] immune response to HIV gag from EP compared to injection only (IO) of plasmid DNA. More recent studies have broadened the list of

pathogens which EP has been successfully used *in vivo* to include other viral pathogens such as: Simian Immunodeficiency Virus [15-18], Severe Acute Respiratory Syndrome [19, 20], Influenza [21-25], West Nile and Japanese Encephalitis [26, 27], as well as Hepatitis B and C [28-32] and Human Papilloma Virus [33, 34]. EP delivered DNA vaccines expressing proteins of the parasitic infection *Plasmodium falciparum*, one of the parasites causing malaria [35], as well as bacterial infections like Bacillus *anthracis [36]*, *Clostridium botulinum [37]*, and *Mycobacterium tuberculosis* [38] have also been demonstrated to enhance immunogenicity. These results demonstrate the capacity of EP to enhance not only gene delivery and protein expression but also its ability to stimulate the host immune response against a wide variety of pathogens.

Current electrically mediated DNA vaccines employ painful invasive needle electrodes that are inserted into the muscle for electrical stimulation. The primary tissue used for *in vivo* EP is muscle because it is accessible, highly vascularized, multinucleated, and expresses DNA for long periods of time due to the post-mitotic nature of the tissue [39]. However, pain associated with administration is not desirable. As such, alternative delivery sites and methods have been explored. The skin is an attractive target for vaccination because of the high proportion of antigen presenting cells (APC) and large surface

area. Recent studies, as well as work done in our laboratory, demonstrated that intradermal electrically mediated DNA expression can be increased both locally and systemically [8, 40-44]. Electrodes developed for skin EP include: caliper, plate, tweezer, and clip electrodes as well as several needle electrodes [14, 45-48].

To develop an electrically mediated intradermal DNA vaccine we utilized the non-invasive multi-electrode array (MEA), shown in figure 1, for EP delivery. The MEA has 16 electrodes placed 2mm apart and is arranged in 4 rows [45]. Pulses are administered in a sequence that utilizes 4 electrodes at a time, forming 2 X 2 mm squares (9 total squares). Pulses are applied in pairs, in two directions, perpendicular to each other (18 pulses) for 4 rounds of pulsing (72 total pulses). While we have not as yet modeled or directly measured the fields generated across the treated area of skin, we believe by applying the field across a smaller area (2 X 2 mm) will facilitate obtaining a more uniform field then would be obtained when the field is applied across the entire treated area (6 X 6 mm). Our lab previously demonstrated that this electrode, when used in a guinea pig skin model, could significantly increase reporter gene activity [45]. Conditions required for optimal expression were determined to be between 200-300 V/cm and 150ms.

An additional consideration for establishing a MEA delivered DNA vaccine is choosing the appropriate animal model. Guinea pig skin is similar to human skin in thickness and morphology [49]. For this reason, we selected the guinea pig model to better evaluate our delivery approach utilizing a small animal model with skin similar to humans. Therefore, the goal of this study was to evaluate intradermal MEA EP delivery of Hepatitis B surface antigen in a human-like skin model.

Methods

2.1 Ethics Statement: All animal procedures were conducted in a facility (USF) that is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Public Health Service (PHS). Research was conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Florida, College of Medicine (protocol # 2879). All animals were housed, handled and utilizing following guidelines of the United States National Institutes of Health.

2.2 Animals: Female Hartley guinea pigs between 200-250g were used in this study to evaluate skin EP conditions. Guinea pigs were housed at the University of South Florida, College of Medicine vivarium and were rested for one week prior to experimentation. Guinea pigs were anesthetized with 2.5-3.0% isoflurane before and during all procedures. No previous exposure to Hepatitis B virus was known.

2.3 Plasmid: The plasmid used in this study was gWiz™ HBsAg (Aldevron, Fargo, ND). This plasmid encodes for the surface antigen of Hepatitis B and is driven by the CMV promoter.

2.4 Immunization: All guinea pigs were intradermally injected with 100µg (2mg/ml) of gWiz™ HBsAg at two sites on the left flank. MEA EP was performed at 300V/cm and 150ms and 72 pulses. The two groups used in this study were control group injection of plasmid only (IO) and injection of plasmid plus EP (I +EP). All groups were boosted with the same condition at Day 14.

2.5 serum collection: Guinea pigs were bled through the jugular vein at various time points from Day 0 through Day 168. Blood was collected and serum isolated in serum separator tubes. Serum was diluted two-fold starting at 1:10.

2.6 Tissue collection: Guinea pigs were treated as described with gWiz™ HBsAg with and without EP. Those guinea pigs whose tissue was collected for plasmid expression were sacrificed 48 hours after one treatment and skin samples were harvested by excising the treatment site and snap frozen. Those guinea pigs whose tissue was collected to assess damage and cell infiltrate were treated and harvested 96 hours after one treatment and the tissue was snap frozen.

2.7 Indirect ELISA for the detection of Hepatitis B surface antigen antibodies: The enzyme linked immunosorbant assay (ELISA) was

used to assess the production of antibodies from treatment and performed per manufacturer's protocol (Aldevron). Briefly, a 96-well plate (Nunc) was coated with 10μg/ml of Hepatitis B surface antigen (Aldevron) and allowed to coat overnight at 4°C. The plate was blocked with 3% BSA in PBST for 2 hours at 37°C. Serum samples were two-fold diluted in blocking buffer and added to the plate for 2 hours at 37°C. Goat anti-Guinea pig-AP antibody was added at a 1:10000 dilution in blocking buffer. AP substrate, pNPP, (Sigma) was added to colorize and the plate was read at 405nm.

2.8 Immunohistochemistry: Pathological analysis of the skin sections was performed to determine the extent of plasmid expression as well as inflammation and tissue damage. An anti-HBsAg was used to detect plasmid expression. Skin samples taken 48 hours after treatment were frozen, sectioned, and placed on slides. Slides were rehydrated and then blocked with 3% BSA in PBST and incubated in a humidifying chamber for 1 hr. A HRP conjugated anti-HBsAg (AbDSerotec) was made in blocking buffer at a 1:200 dilution. All samples were counterstained with Hematoxylin& Eosin. Samples collected at 96 hours frozen, sectioned, and placed on slides were stained with H & E to determine the extent of cellular infiltrate/inflammation.

2.9 Statistical analysis: All Guinea pigs were bled at Day 0 to determine background optical density (OD). OD's were averaged and 2 standard deviations added to determine positive (0.1 OD). Experimental serum samples were diluted two-fold starting at 1:10. End point titers were calculated and plotted as Geometric Means. Significance was determined by student t-test using the bonferroni correction for multiple comparisons.

Results

3.1 Plasmid expression from EP.

The first step in evaluating the MEA for delivery of DNA vaccines in a human-like model was to determine the expression levels of gWiz™ HBsAg. Guinea pigs were treated as described with or without EP using the MEA. 48 hours after delivery the guinea pigs were euthanized and the treated skin harvested and processed for histological evaluation. Expression of HBsAg was determined by immunohistochemistry. Expression of HBsAg is seen in IO and I+EP (Fig 1a and b), however increased staining was observed in the I+EP samples. Expression is contained within the epidermis of IO animals. When compared to I+EP animals expression can be seen within the epidermis and dermis.

3.2 Immune cell infiltrate and tissue damage

To determine whether EP with the MEA would recruit immune cells to the treatment site and cause inflammation, guinea pigs were treated as described and tissue samples harvested 96 hours after treatment. Samples were stained with H&E to assess cellular infiltrate, damage, and necrosis from treatment. The induction of immune

stimulation is important for vaccines in general, but can be limited for DNA vaccines. Induction of immune cell infiltrate was observed (Fig 2 C-F 100X magnification). Background levels, Fig 2c, of infiltrate are demonstrated in no treatment control and correspond to low levels of cellular infiltrate (purple). IO samples show slight increases in infiltrate as compared to no treatment, Fig 2d. In contrast, I+EP samples show a large increase in cellular infiltrate, Fig 2e. I + EP groups contained primarily macrophages and multi-lobed cells, most likely activated neutrophils (200x magnification Fig. 2f), corresponding to a prolonged inflammatory immune response [50].

Edema was seen in all samples except no treatment controls; and did not appear increased due to EP. This is most likely a result from the injection of plasmid into the tissue. In most samples tissue damage and necrosis were not seen. However, two EP delivered samples had minimal ulcerations at 96 hours after treatment, one of which also had about 1% necrosis. There were no other samples showing damage or necrosis (data not shown). Gross evaluation of the skin shows no difference between IO and I+EP groups over time (Fig 3). Complete visual recovery of the skin is seen by Day 7.

3.3 Anti-Hepatitis B surface antigen antibodies

While cellular infiltrate can be an early indicator of immunity, a more accurate measure is the induction of specific antibodies generated against HBsAg. Anti-HBs were measured by ELISA over time. Guinea pigs, treated and serum collected as described in methods, showed significant increases in antibody expression from three weeks after initial treatment through week 24. The data collected was from 3 independent experiments (n=6 for each experiement) with a total n of 18 for both IO and EP groups. Peak expression for both groups occurred at week 18 with IO groups having a GMT of 1000 and I+EP animals at 5000 (Fig 4). The fold increase over IO remained relatively constant at about 5 fold with the greatest fold increase over IO of 6.5 occurring at week 18.

Discussion

These data demonstrate that the MEA can be effective for the use in electrically mediated DNA vaccination in a human-like skin model. EP with the MEA generated increased plasmid expression as well as an increase in immune infiltrate after treatment. The magnitude of immune infiltrate was greater in EP groups than IO and there was minimal to no skin damage associated. Specific, lasting, and significant levels of antibodies were greater than IO. This is the first report to demonstrate the use of the MEA for DNA vaccination in a human-like skin model.

DNA vaccination is advantageous because it does not integrate into the host DNA, it is cost effective to produce and easily stored, it can be highly specific for tissue and/or cell type and can be made to vaccinate against multiple agents simultaneously. The skin is an ideal target for DNA vaccination due to the large surface area and presence of antigen presenting cells like langerhan's and dermal dendritic cells, specialized for induction of immunity [51]. However, injection of plasmid alone does not induce high enough immune responses to be protective. EP is one method that has been shown to increase both plasmid expression as well as immunity.

Previous EP methods have involved painful penetrating electrodes that go into the muscle to facilitate delivery. Further advancements have been made using non-penetrating electrodes such as caliper and plate electrodes. However, these electrodes require high voltages to enhance delivery and therefore can cause tissue damage. In this study, we have evaluated a non-penetrating electrode which reduces the gap width between electrodes to 2mm thereby reducing the absolute voltage applied and preventing visible tissue damage while still increasing plasmid expression and immunity.

As expected from our previous publication [45], EP with the MEA enhanced expression. While the exact reason for the effectiveness of EP remains unknown, increased plasmid expression at least in the case of DNA vaccination, plays an important role in recognition by the immune system [52]. EP has been shown to have an adjuvant effect by recruiting immune cells to the site of pulse application [53]. In our study, we saw an influx of nucleated cells from EP treated samples. These cells are most likely neutrophils and macrophages based on morphology. This is most likely a combination of both an EP mediated adjuvant effect and increased plasmid expression. The induction of macrophages and polymorpho-nucleated neutrophils is indicative of a chronic inflammatory response. While the perception of prolonged inflammation is typically negative in our case it indicates that the

expression of the plasmid is present for a prolonged period of time, giving the immune response enough time to perform its function. Based on our earlier work [45] we would expect this prolonged expression to decrease after approximately 14 days, therefore allowing the body to heal and not generate deleterious effects from inflammation.

These findings seem to correlate with our antibody data, where an increase in the presence of specific antibodies was measured over time. These antibodies were significantly increased as compared to injection only. Geometric mean titers ranged from 4000-16000 peaking at week 18. Antibody levels remained elevated until dropping off after week 21, but still remained increased as compared to injection only. The enhanced intensity of humoral immunity by EP with the MEA corresponds to previously published skin EP results [54-57]. One of the primary reasons for evaluating our delivery method with Hepatitis B was because it is a well characterized vaccination model. Published studies have reported geometric mean titers in conjunction with protective efficacy in guinea pigs. While the presented GMT's in these papers were higher than ours, they also reported protective levels more than 100 fold above the necessary levels. Our GMT's are likely to still be within the protective range without generating unnecessary additional responses [58, 59].

Comparing specifically to Hepatitis B DNA vaccines delivered by EP several animal models have been evaluated and EP has been shown to have protective levels from 10-1000mIU/ml [6, 9, 32, 60, 61]. The most recent comparable publication evaluated a minimally invasive device for protective vaccination against influenza [62]. While their results were only presented as neutralizing titers against flu and cannot be compared directly we believe that our electrode design generates immune responses of equal quality without tissue penetration.

The data represented here demonstrate the capability of the MEA to increase plasmid expression, immune cell infiltrate and inflammatory response, as well as antibody production over 24 weeks in a human-like skin model. This information presents a potential new method for DNA vaccination that may be translatable to humans. Further studies will examine the MEA for use in DNA vaccination against other infectious agents.

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Figures

Figure 1.

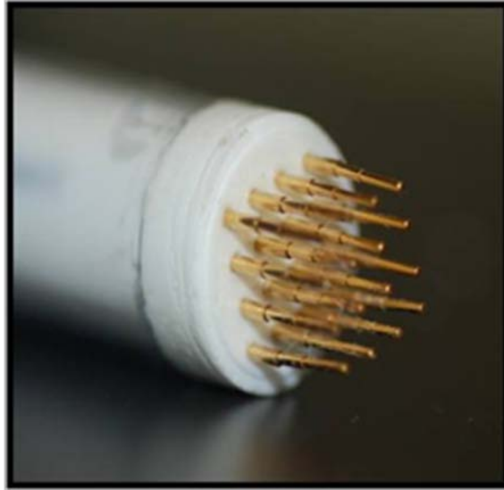

Figure 2.

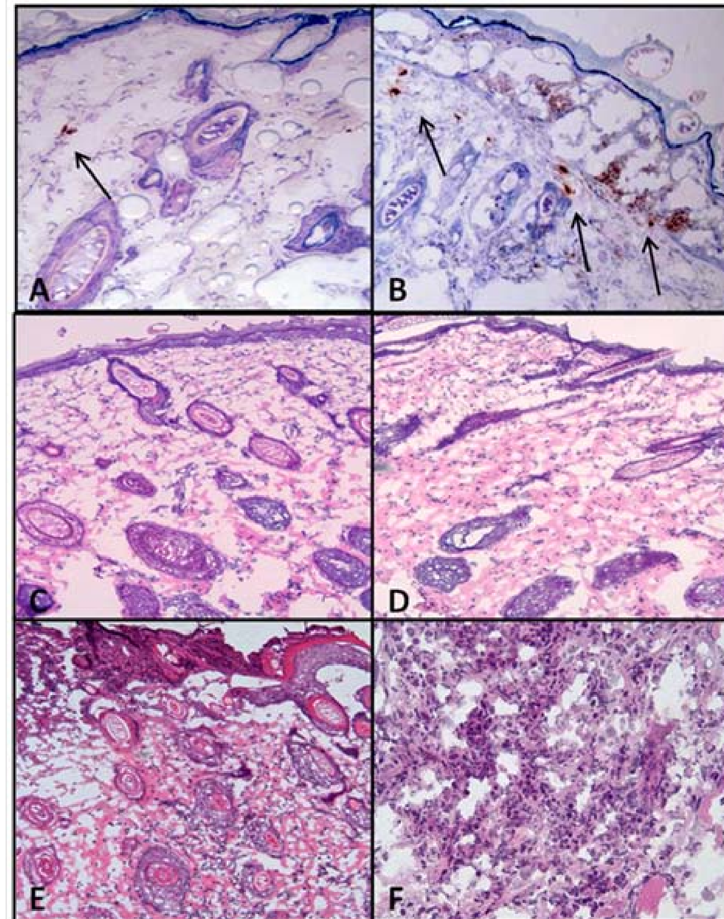

Figure 3.

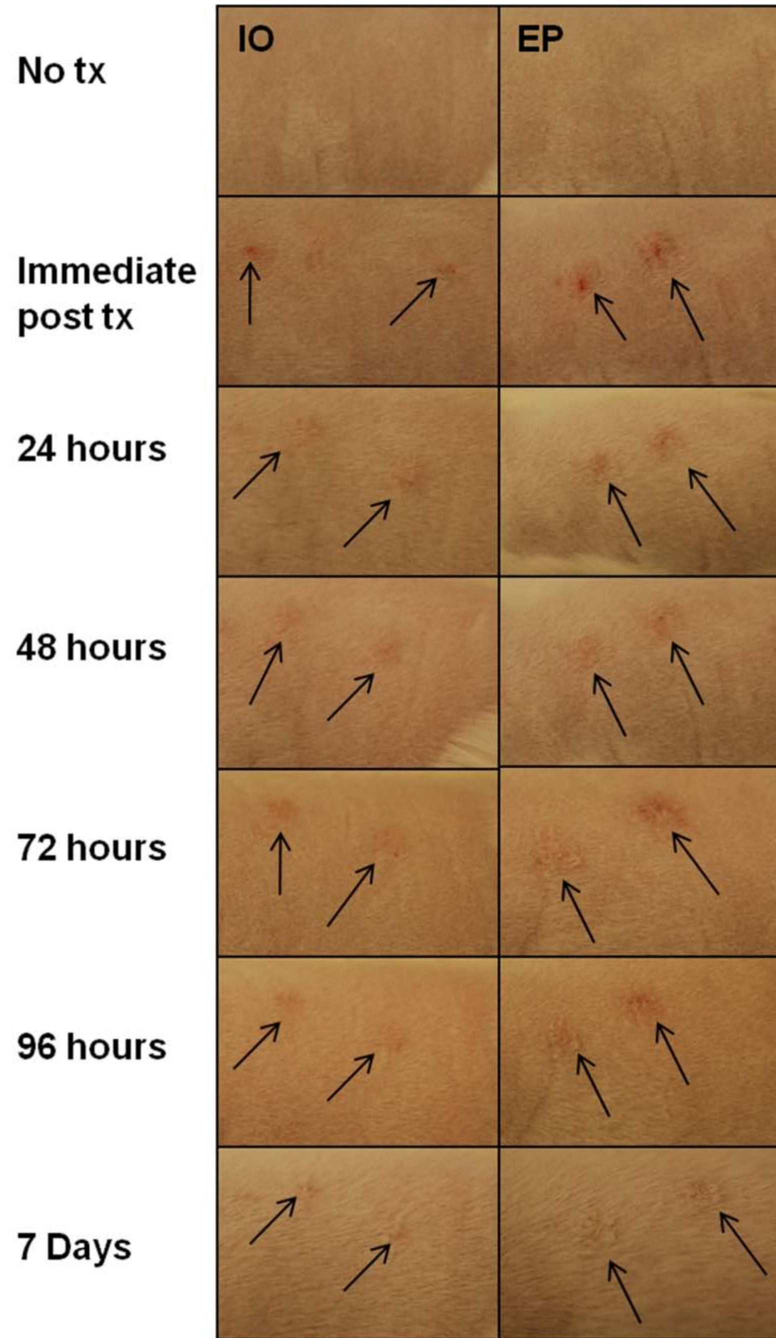

Figure 4.

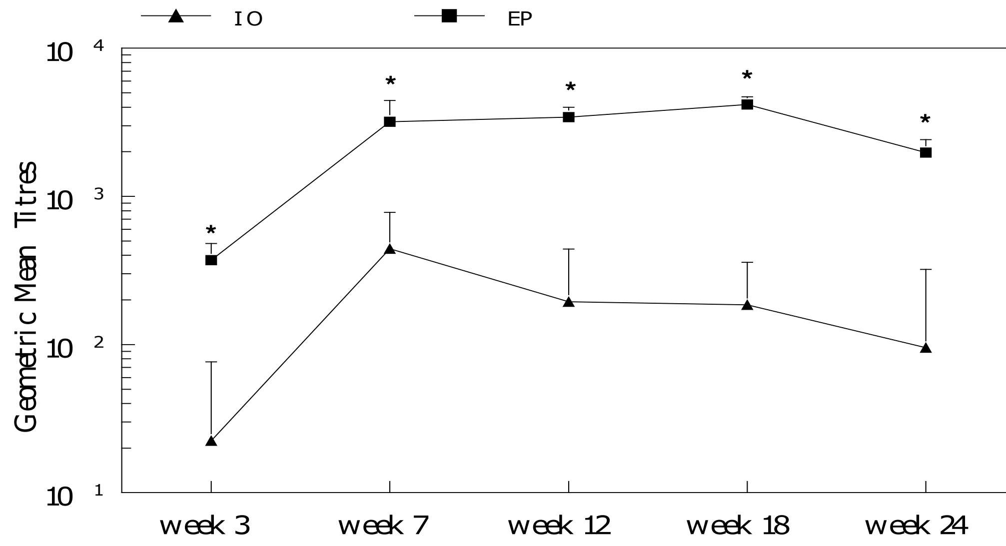

Figure legends

Fig. 1 Non-invasive Multi-Electrode Array. The MEA has 16 electrodes placed 2mm apart and is arranged in 4 rows. Pulses are administered in a sequence that utilizes 4 electrodes at a time, forming 2 X 2 mm squares (9 total squares). Pulses are applied in pairs, in two directions, perpendicular to each other (18 pulses) for 4 rounds of pulsing (72 total pulses). This image is reprinted from The Journal of Controlled Release doi:10.1016/j.jconrel.2011.01.014 Siqi Guo, Amy Donate, Guarav Basu, Cathryn Lundberg, Loree Heller, Richard Heller "Electro-gene transfer to the skin using a non-invasive multi-electrode array" with permission from Elsevier.

Fig. 2 Plasmid expression and inflammation in the skin.
Guinea pigs were treated as described in Methods 2.1 with pHBsAg. Expression of plasmid was evaluated at 48 hrs post treatment by IHC (A-IO; B I + EP). Inflammation was measured 96 hrs post treatment and assessed by H&E (C-No treatment; D- IO, E – I + EP) at 100X magnification and 200X magnification (F- I + EP).

Fig. 3 Visual assessment of skin damage and healing.

Guinea pigs were treated as described in Methods 2.1 with pHBsAg with or without EP. Images were taken of skin pre treatment, immediately post treatment, and at 24, 48, 72, 96 hours and at 7 days. Arrows indicate the treatment sites.

Fig. 4 Evaluation of anti-HBs serum titer.

Guinea pigs were treated as described in Methods 2.1 with pHBsAg. Serum was collected at multiple time points and an ELISA performed. Geometric mean titers are expressed. Positive was determined by two standard deviations greater than the Day 0 OD. IO and EP n=6 for each experiment with 3 independent experiments conducted (total n=18). Statistics were determined by two-sided student t-test with bonferroni correction to $p<0.05$.

About the Author

Amy Donate received her Bachelor's degree from the University of Florida in the spring of 2005. In 2006 she received her Master's degree in Medical Microbiology at the University of South Florida College of Medicine. She conducted research for her Master's thesis with Burt Anderson Ph.D. on the regulation of the VirB operon of *Bartonella henselae*. In 2006 she entered the doctoral program in Molecular Medicine at the University of South Florida, College of Medicine. While researching for her dissertation, she received a predoctoral fellowship in Multidisciplinary sciences. She was awarded a travel grant at the Gordon Research Conference in 2008. Amy was selected to be an oral abstract presenter at the American Society for Gene Therapy also in 2008 and received a poster award at the International Symposium on Bioelectrochemistry in 2010.