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Research Article

DNA Vaccines Delivered by Microneedle and Tattoo Gun Induce Protective Immune Responses to HLA-A2.1 Restricted CRPV E1 and HPV16E7 Epitopes in HLA-A2.1 Transgenic Rabbits

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Article Information

Received date: Mar 24, 2016
Accepted date: Jun 02, 2016
Published date: Jun 06, 2016

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Keywords DNA vaccine; HLA-A2.1 transgenic rabbit; Gene gun; Tattoo gun; Microneedle; Delivery; CRPV; Protection; Immune response; *In vivo* killing

Abbreviations CRPV-Cottontail Rabbit Papillomavirus; HPV-Human Papillomavirus

Abstract

Objective: We assessed two novel and cost-effective DNA delivery methods as alternatives for our well-established gene gun delivery system, using a preclinical rabbit papillomavirus model.

Methods: HLA-A2.1 transgenic rabbits were immunized with CRPVE1ep1-5 or HPV16E7/82-90 epitope DNA vaccines via gene gun, tattoo gun or microneedle and challenged with either wild type CRPV or CRPV containing HPV16E7/82-90. The tumor outgrowth were monitored and recorded weekly. *In vivo* killing was conducted in tattoo gun and microneedle vaccinated animals.

Results: Tattoo gun delivery provided comparable protection with both DNA vaccines when compared with gene gun. Microneedle provided similar protection as tattoo gun. Specific *in vivo* killing was detected in CRPVE1ep1-5 DNA vaccinated animals by both tattoo gun and microneedle.

Conclusion: Both tattoo gun and microneedle can be used as alternatives for the gene gun for DNA vaccination. These two methods are more cost-effective and microneedle minimized pain in animals.

Gene gun delivery of DNA vaccines has proven to be effective for protective and therapeutic immunization in various animal models. However, gene gun vaccine components are expensive, and maintenance and repairs for the gene gun are costly. Two novel delivery methods – the tattoo gun and microneedle - have been reported promising for DNA vaccination. Tattoo gun allows for direct application of the DNA vaccine to the skin followed by electronic stimulation, and microneedle is noninvasive and generates minimal pain in the host. However, these two delivery systems have not been tested in the rabbit model before. In the current study, we performed experiments comparing these three vaccination strategies using two HLA-A2.1 restricted epitope DNA vaccines (HPV16E7/82-90 and CRPVE1ep1-5 epitope), which have been previously shown to generate robust protective immunity via gene gun delivery. In the first study, we compared gene gun with tattoo gun side by side using the HPV16E7/82-90 DNA vaccine. Comparative protection was found in these two methods. In the second experiment, we compared microneedle with tattoo gun delivery using the CRPVE1ep1-5 epitope DNA vaccine. DNA vaccination by both tattoo gun and microneedle resulted in significantly smaller tumors when compared with those of the control group [$P<0.05$, one way ANOVA analysis]. *In vivo* killing assay demonstrated that animals immunized via both microneedle and tattoo gun showed increased specific killing to the epitope presenting cells. These data indicate that DNA vaccination via microneedle and tattoo gun is simple, useful, and cost-effective alternative to the gene gun and produces comparable results.

Introduction

DNA vaccines show promise as prophylactic and therapeutic strategies against chronic viral infections [1-3] and antigen-specific tumors [4]. DNA vaccines spontaneously transfect cellular targets and continue to express vaccine antigens, which may aid the induction of immunologic

memory [3,5-7]. Additionally, DNA vaccines are relatively stable, and their production can be easily scaled up [5]. One hindrance to DNA vaccination is that traditional delivery methods, such as intramuscular (i.m.) and intradermal (i.d.) injection, are inefficient at generating a strong immune response even at high DNA vaccine concentrations [8]. Consequently, other methods of DNA vaccination, such as particle-mediated epidermal delivery, tattoo gun, and recently adopted microneedle delivery have been developed to improve the immunogenicity of DNA vaccines [9,10]. Gene gun immunization uses gold particles as a carrier for DNA molecules. The DNA-coated gold particles are driven into the epidermal layers of the skin at high pressure, delivering the DNA to keratinocytes and skin-resident Dendritic Cells [DCs] [11-13]. Gene gun-mediated DNA vaccination invokes a stronger immunological response than traditional delivery methods in some animal models including mice [14,15], rabbits [16,17], and non-human primates [18,19]. Gene gun immunization also generates both humoral and cellular immune responses in human clinical trials [4,12,20]. DNA vaccination via tattoo gun uses a vibrating solid needle to deliver the DNA vaccine directly to the epidermal layers of the skin through multiple tiny puncture wounds. This method transfects cells in the epidermal layers of the skin and promotes T cell activation [21]. DNA tattoo gun induces a stronger immune response in both mice and non-human primates than traditional intramuscular vaccination [22,23]. Microneedle delivery has been found to be effective for DNA and peptide immunization [24-26].

When compared side by side, both gene gun-mediated and tattoo gun-mediated DNA vaccination were equally effective in generating strong antigen-specific T cell responses against an HPV16E7 epitope in vaccinated mice [5,27,28]. HPVs induce hyperproliferative lesions at both cutaneous and mucosal sites, and it is well recognized that "high risk" HPV types are the etiological agents of cervical cancer. The current commercially available prophylactic Virus-Like Particle (VLP) vaccines induce protective humoral immune responses [29], but alternative vaccines and vaccination strategies that induce cell-mediated immune responses are necessary for therapeutic intervention of established HPV disease [30]. Our laboratory uses the Cottontail Rabbit Papillomavirus (CRPV) rabbit model to study host immunity induced during a natural PV infection. Previously, our laboratory demonstrated that cell-mediated immunity to viral proteins E1, E2, E6, E7, E8 and L1 [31-36] is promoted in the CRPV rabbit model by gene gun-mediated DNA vaccination. Additionally, gene gun-mediated DNA vaccination of our established HLA-A2.1/CRPV transgenic rabbit provokes strong protective immune responses to the well-known HPV16E7 82-90 epitope [37] and computer-predicted HLA-A2.1-restricted epitopes from the CRPVE1 gene *in vivo* [32,38]. However, the expenses incurred from the components necessary for gene gun-mediated DNA vaccination and gene gun repair, as well as the time loss during said repairs, prompted our laboratory to search for an alternative DNA vaccination method. In these studies, we demonstrated the protective immunity induced in HLA-A2.1 transgenic rabbits via tattoo gun and microneedle delivery. Initially, we determined that a GFP-expressing plasmid could be introduced successfully to the inner ear skin of rabbits using these three delivery methods. Then, we gene gun and tattoo gun delivery using the HLA-A2.1-restricted HPV16E7 82-90 epitope DNA vaccination in HLA-A2.1 transgenic rabbits followed by challenge with modified CRPV DNA genomes containing this same epitope embedded in the CRPV E7 gene. The tattoo gun was as effective as the gene gun in generating protective cell- mediated immune responses in the HLA-A2.1/CRPV rabbit model. Subsequently, we compared microneedle with tattoo gun delivery by immunizing HLA-A2.1 transgenic rabbits with a multi-epitope (CRPVE1/ep1-5) DNA vaccine following the wild type CRPV infection. Microneedle delivery was found to be comparable to the tattoo gun in protecting animals against viral infection. Both microneedle and tattoo gun delivered DNA vaccine stimulated specific *in vivo* killing in corresponding animals. In summary, both tattoo gun and microneedle delivery systems are attractive alternative vaccination strategies to gene gun-mediated particle delivery in the HLA-A2.1/CRPV rabbit model.

Materials and Methods

DNA Vaccines

The HPV16E7/82-90 epitope DNA vaccine was designed with 5 repeats of the single epitope separated by Alanine-Alanine-Tyrosine (AAY) spacers [39]. A universal Tetanus Toxoid [TT] T-helper motif [40] followed an N-terminus Kozak sequence, and an ubiquitin motif at the C-terminus was also included in the synthetic sequence as described earlier [38]. The complete vaccine sequence was then cloned into the expression vector pCX (Invitrogen). The finished vaccine product was designated HPV16E7/82-90 epitope DNA vaccine [39]. The CRPVE1ep1-5 epitope DNA vaccine was designed as previously described [38]. DNA vaccines were isolated and purified using the QIAGEN Maxiprep plasmid isolation kit. The DNA vaccines delivered using the gene gun were adjusted to a plasmid concentration of 1ug/ml in 1×TE. The DNA was then precipitated onto 1.6μm-diamter gold particles at a ratio of 1μg of DNA/0.5mg of gold particles as described by the manufacturer (Bio-Rad, Hercules, California). The DNA vaccines delivered using the tattoo gun and the micro needles were subjected to Cesium chloride density gradient centrifugation after maxiprep isolation and their concentrations were adjusted to 500ng/μl in 1×TE.

DNA Plasmids

The Enhanced Green Fluorescent Protein (EGFP) cloned into the mammalian expression vector pCR3 (Invitrogen) was used to detect DNA delivery. H.CRPV construct cloned into a pUC19 vector using the SalI site found in the L2 gene of CRPV at base pair position 4572 was used as wild type CRPV [41]. A ClaI site was added to H. CRPV at base pair position 1382, just downstream of the E7 stop codon [42]. A subclone of the CRPV E7 gene cloned into a pUC19 vector between the EcoRI and ClaI sites underwent a single round of site directed mutagenesis to insert the HPV16 E7 82-90 [LLMGTLGIV] sequence in frame into the CRPV E7 gene just upstream of the E7 stop codon. Primer sequences used for this single step mutagenesis were 5'-GCC-CGG-AGT-GTT-GTA-ACC-TGC-TGA-TGG-GCA-CCC-TGG-GCA-TCG-TGT-GAA-AAT-GG-CTG-AAG-GTA-CAG-ACC-3' and 5'-GGT-CTG-TAC-CTT-CAG-CCA-TTT-TCA-CAC-GAT-GCC-CAG-GGT-GCC-CAT-CAG-CAG-GTT-ACA-ACA-CTC-CGG-GC-3' with the underlined portion indicating the inserted nucleotide sequences. This procedure produced a CRPV E7 gene containing nine additional amino acids at the C-terminus, which was confirmed by DNA sequencing. After cloning the modified CRPV E7 gene into the H.CRPV construct using the EcoRI and ClaI sites, the sequence of the new genome, CRPV/E7ins82-90, was confirmed

by DNA sequencing. To create the tandem repeat genome used for infection, the CRPVE7ins82-90 genome was digested with SalI, liberating the viral DNA from the pUC19 vector. A second construct containing nucleotides 1063 to 4575 of wild type CRPV cloned into pUC19 between the SalI and EcoRI sites was digested with SalI. The two DNA restriction products were then ligated creating a new modified CRPV genome clone that contains the entire CRPV/E7ins82-90 genome plus an additional 3523bp piece of CRPV that includes the wild type CRPV E7 gene as well as wild type CRPV E1, E2, E4 and parts of the E5 and L2 genes. The CRPV/E7 (82-90) TR genome sequence was confirmed by DNA sequencing and orientation was established by digestions with restriction enzymes SphI, Aat2, and HindIII. DNA plasmids were isolated and purified according to the Qiagen maxiprep plasmid isolation kit protocol. The concentration of the pCR3-EGFP plasmid that was delivered using the gene gun was adjusted to 1μg/μl. Viral DNA plasmids and the pCR3-EGFP plasmid that were applied using the tattoo machine were subjected to an additional purification step through cesium chloride density gradient centrifugation. The concentrations of viral DNA plasmids were adjusted to 200ng/μl in 1× TE and the concentration of pCR3-EGFP was adjusted to 500ng/μl in1× TE.

Rabbit Vaccination and DNA Challenge

Outbred HLA-A2.1 transgenic rabbits were maintained in the Pennsylvania State University College of Medicine animal facility. Outbred non-transgenic New Zealand White rabbits were purchased from Covance Research Products, Inc. The Institutional Animal Care and Use Committee approved all animal care and handling procedures used in all animal studies. Rabbits were divided into groups and were vaccinated three times at three-week intervals with either the CRPVE1ep1-5 DNA vaccine or the HPV16E7/82-90 epitope DNA vaccine. Prior to DNA vaccination by either strategy, the inner ear skin of each rabbit was cleaned with 70% ethanol. For gene gun-mediated particle delivery, the ear skin was barraged with DNA coated gold particles at a rate of 400lb/in2 from a helium driven gene gun as described previously [16]. Each rabbit received a vaccine dose of 20 shots (theoretically 20μg) at each immunization. For DNA vaccination by tattoo gun, vaccines were delivered to the inner ear skin of each rabbit with a 14-bundle linear tattoo needle and a commercially available rotary tattoo machine (NMT-2 NeoTat Linear Series tattoo machine, taptatdaddio.com). The DNA was delivered to a depth of 1-2 mm as described [21]. A template of 2cm ×1cm was used as a surface area guide to ensure consistency between animals. Each area was tattooed 30-times at 2 second intervals at a voltage of 15 V (NPS-15 DC power supply, taptatdaddio.com). This voltage setting corresponds to approximately 144 hits per second. Therefore, each rabbit ear received a total number of 120,960 needle punctures at every immunization. A 10μg dose was delivered to each ear for a total dose of 20μg per vaccination per rabbit. Minor mechanical trauma, swelling and oozing of serous fluid was observed after tattoo gun delivery. For microneedle delivery, we first prepared DNA coated microneedles (Microneedle Systems, LLC, GA). 30ul DNA (3μg/μl) was loaded in the coating well and two microarrays taped together and dipped into the well for three times for two minutes (allow drying for 5 minutes in between dips). The DNA coating efficacy was tested by dissolving the two microarrays into 1ml ddH_2O and measured by Nanodrop. DNA coated microneedles were put into the inner ear skin (shaved and 70% ethanol wiped) for 2 minutes and add 20ug of DNA and poke 5 times with the microneedles to work the DNA in (wait for 2 minutes). Light bleeding was observed in some animals. Four days following the final booster, rabbit backs were shaved and scarified as described [43]. One week after the final booster rabbits were challenged with wild type CRPV DNA at a dose of 5μg/site in a 50ul or with CRPV/E7ins82-90 and CRPV/E7 (82-90) TR at a dose of 10μg/site in a 50ul volume and wild type CRPV DNA as described above. Rabbits receiving the pCR3-EGFP DNA plasmid were immunized in the ears with 10μg of plasmid DNA using the gene gun or the tattoo gun. Ear punch biopsies were taken 24 hours later and subjected to histological analysis as described elsewhere.

Tetramer Binding Assay

Spleen cells from all rabbits were harvested at the termination of the experiment. 0.5×106 cells from each rabbit were washed and cultured in RPMI with 5% autologous serum together with human recombinant IL-2 as described previously [32]. After two in vitro stimulation with autologous cells pulsed with corresponding peptide, the bulk CTLs were analyzed with corresponding tetramers (generous prepared by the NIH tetramer facility). The tetramer positive CD8 T cells were gated and calculated automatically with cytometry analysis in the core facility of Pennsylvania State University College of Medicine.

In vivo Killing Assay

Rabbit Peripheral Blood Lymphocytes [PBLs] were isolated with lympholyte®-mammal (Cedarlane) from 10ml fresh blood collected from the DNA vaccinated animals before viral DNA infection. Half of the PBLs were pulsed with either test peptide (CRPVE1/303-311) or control peptide (HIV gag/pol 17/77-85) (1μM) at 37°C for 1 hour. The peptide- pulsed PBLs were subsequently labeled with either 2.5 μM or 0.25μM of carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). The labeled cells were counted and infused back into the matched animals. The next day the labeled cells were retrieved from the test animals' PBLs and were analyzed by flow cytometry. The ratio of input *vs* retrieved labeled cells was used as the indicator of specific killing in these vaccinated animals.

Histology and Immunofluorescence Detection

One day after GFP-expression plasmid delivery via gene gun, tattoo gun and microneedle, ear punch biopsies were collected and cryo-preserved tissue samples were subjected to fluorescence and bright field microscopy. Fresh tissue 3 mm punch biopsies were snap frozen in liquid nitrogen and embedded in Optimal Cutting Temperature (OCT) medium (Sakura Finetek, Torrance, CA). Sequential cryostat sections of 6-7 um were mounted on silane coated glass slides and stored at -20°C. The first section was subjected to fluorescence microscopy using a Nikon Eclipse E600 microscope for detection of GFP. The next section was stained for DNA with Hoechst 33342 (Molecular Probes) and visualized using the same Nikon Eclipse E600 microscope. The third sequential section was fixed, stained with hematoxylin and eosin, and subjected to bright field microscopy. All images were photographed and digitally prepared using Adobe Photoshop in an identical manner.

Statistical Analysis

Papilloma size was determined as described [44]. Briefly, the cubic root of the product of length, width, and height in millimeters

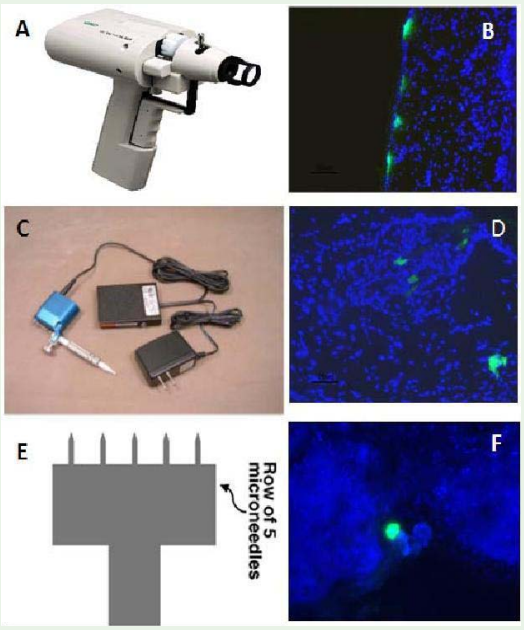

Figure 1: Detection of EGFP expression in ear punch biopsy 24 hours after 10ug of pCR3-EGFP was delivered using the gene gun [A, 10×], the tattoo gun [C, 10×], and the microneedle [E, 44610×]. Ear punch biopsies were collected and cryo-preserved in OTC medium. Slices were cut and prepared slides were stained briefly with Hoechst. The slides were visualized to determine successful plasmid delivery. GFP expressing cells can be identified under the fluorescent microscope as shown in B [10×], D [20×] and F [10×] respectively.

of individual papilloma was calculated to determine the Geometric Mean Diameter (GMD). Measurements were gathered weekly starting 3 weeks after viral DNA challenge. The data are represented as the means (+/- standard errors) of the geometric mean diameters for each rabbit group. One way ANOVA and unpaired t-test comparisons were used to assign statistical significance ($p < 0.05$ was considered statistically significant). The protection rates were calculated as previously described [38] and the Fisher's exact test was used to determine statistical significance.

Results

Detection of EGFP Gene Expression After DNA Plasmid Delivery to the Inner Ear Skin of New Zealand White Rabbits by A Gene Gun, A Tattoo Gun and A Microneedle Array

Our laboratory has used gene gun-mediated vaccination to deliver DNA plasmid vaccines to NWZ white rabbits with great success [16,31-33,35,36,45,46]. To confirm that a DNA plasmid could be efficiently delivered using a tattoo gun and a microneedle, an EGFP-expression plasmid was employed. GFP expression in the epidermis and dermis layers of the skin was detected in tissue samples from rabbits receiving the DNA plasmid by gene gun (Figure 1A and B), tattoo gun (Figure 1C and D) and microneedle (Figure 1E and F).

Gene Gun and Tattoo Gun-Mediated Vaccinations Provided Similar Levels of Protection against Challenge with a Modified CRPV Genome Containing the HPV16e7 82-90 Epitope Inserted in the E7 Gene

Our previous studies demonstrated that HPV16E7/82-90 epitope DNA vaccination provided specific and protective immunity in HLA-A2.1 transgenic rabbit [39]. In the current study, we applied this epitope DNA vaccine to test whether tattoo gun delivery would generate equivalent protection when compared with the gene gun delivery. Both HLA-A2.1 transgenic and non-transgenic control rabbits were vaccinated three times with the HPV16E7/82-90 epitope DNA vaccine at three-week intervals using either the gene gun or the tattoo gun and subsequently challenged with a hybrid CRPV/E7ins82-90 DNA, into which HPV16E7/82-90 was inserted at the carboxyl terminal of CRPV/E7. Similar levels of protection against challenge with the modified CRPV genome was observed in both HLA-A2.1 rabbit groups immunized with the HPV16E7/82-90 epitope DNA vaccine (Table 1). In contrast, little to no protection was observed in non-transgenic control rabbits receiving the epitope vaccine by either delivery route (Table 1). Additionally, using either method, the mean papilloma size of the immunized HLA-A2.1 transgenic rabbits was significantly smaller than that of the control rabbits (Figure 2).

DNA Vaccination by a Tattoo Gun Yielded Complete Protection Against Infection With a More Vigorous Tandem Repeat [TR] CRPV Genome Containing A Modified CRPV E7 Gene

Our laboratory has shown that HLA-A2.1 transgenic rabbits have a higher level of natural immunity to CRPV infections when compared to non-transgenic rabbits [42]. The modified genome, CRPV/E7ins82-90, produces smaller and slower growing papillomas than the wild type CRPV genome (unpublished observation). Given the trauma by tattoo gun delivery, we want to make sure that the protection observed using the tattoo gun is not due to the natural immunity overwhelming an infection. We created a more vigorous

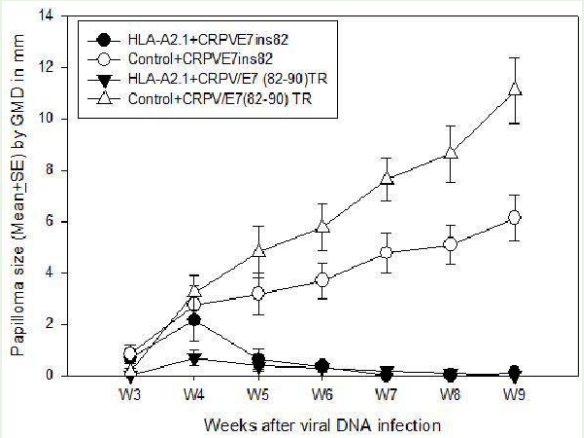

Figure 2: Tumor growth after viral infection in HLA-A2.1 transgenic rabbits [N=3/ group] or control animals vaccinated with HPV16E7/82-90 DNA vaccine via tattoo gun delivery method. After three vaccinations with a three-week interval, the animals were challenged with 5 μg hybrid CRPVE7 ins82 or CRPVE7/E7 (82-90) TR DNA. The tumor growth was monitored weekly and significantly smaller papillomas induced by both hybrid CRPV infection were found in vaccinated HLA-A2.1 transgenic rabbits when compared with those from the control animals after week 5 [$P<0.05$, one way ANOVA analysis]. No significant difference was found between the two hybrid constructs after DNA vaccination in HLA-A2.1 transgenic rabbits [$P>0.05$, one way ANOVA analysis].

Table 1: Protection rate of HLA-A2.1 transgenic and normal rabbits immunized with HPV16E7/82-90 epitope DNA vaccine either by Gene gun or Tattoo gun.

Group	Rabbits	DNA Delivery	Challenged Sites	Protection Rate (%)[a]
1 (N=3)	HLA-A2.1	Gene Gun	18	18/18 (100)[b,c]
2 (N=3)	Control		18	1/18 (6%)
3 (N=4)	HLA-A2.1	Tattoo Gun	12	11/12 (92%)[d]
4 (N=3)	Control		9	0/9 (0%)[e]

Table 2: Tumor protection rates in HLA-A2.1 transgenic and normal New Zealand White rabbits after HPV16E7/82-90 epitope DNA vaccination via Tattoo gun.

Group	Rabbits	Challenged sites	Protection Rate (%)[a]
1 (N=4)	HLA-A2.1	12	12/12 (100)[b]
2 (N=3)	Control	9	0/9 (0)

[a]Protection rate=Papilloma-free sites/Challenge sites; [b]P=0.01 *vs.* the control group, the Fisher's exact test.

Table 3: Tumor outgrowth and tetramer positive T cells from cultured spleen cells *in vitro*.

Group	Rabbit	Vaccine	CRPVE1/303-311 tetramer CTLs of total population (%)	Tumor rates (tumor sites/ infection sites)
A (N=4)	R4265 R4276 R4284 R4268 Total	CRPVE1ep1-5 DNA vaccine via tattoo gun	3.36 0.38 8.67 ND	0/6 6/6 0/6 0/6 6/24 (25%)*[a]
B (N=4)	R4266 R4277 R4286 R4269 Total	CRPVE1ep1-5 DNA vaccine via microneedles	10.71 2.17 1.07 ND	0/6 5/6 0/6 4/6 9/24 (38%)*
C (N=3)	R4278 R4287 R4274 Total	HPV16E7/82-90 DNA vaccine via microneedles	1.44 0.38 ND	6/6 4/6 6/6 16/18 (89%)

*P<0.05 *vs.* control group C, [a]P>0.05 *vs.* group B, the Fisher's exact test, ND-not done.

hybrid CRPV genome that contains a tandem repeat genome with the HPV16E7/82-90 (CRPV/E7 (82-90) TR). The growth rate of papillomas produced by this tandem repeat genome was improved when compared with the previous hybrid construct although it is slower than the wild type CRPV (Figure 3). We repeat the previous experiment except challenging the rabbits with CRPV/E7 (82-90) TR genome. HLA-A2.1 transgenic rabbits were completely protected against challenge with the CRPV/E7 (82-90) TR genome at the termination of the experiment [2]. The mean papilloma size of the papillomas that did grow in the early weeks after the viral challenge was significantly smaller in these HLA-A2.1 transgenic rabbits (Figure 2).

DNA Vaccination by Microneedle Provided Strong Protection that was Comparable to Tattoo Gun Delivery in HLA A2.1 Transgenic Rabbits

Although tattoo gun vaccination stimulated similar protective immunity to the gene gun vaccination, this method also generated local trauma to the hosts which would limit its application to human populations in the future. We have been searching for a noninvasive

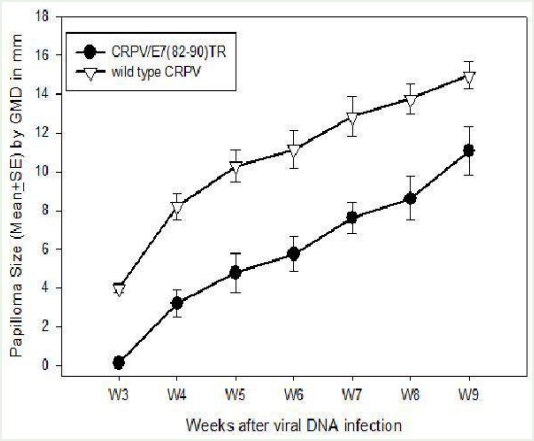

Figure 3: Tumor growth after either wild type or CRPVE7/E7 (82-90)TR DNA infection in 462HLA-A2.1 transgenic rabbits [N=3/ group]. The animals were challenged with 5 μg wild type or CRPVE7/E7 (82-90) TR. The tumor growth was monitored weekly. Significantly smaller papillomas were found by CRPVE7/E7 (82-90) TR DNA infection when compared by the wildtype CRPV [P<0.05, one way ANOVA analysis].

DNA delivery system for many years. A novel microneedle delivery system was reported and was not made available commercially until more recently [25,47]. This noninvasive method has proven to be effective and, most importantly, almost painless to humans [48]. Several animal studies have demonstrated microneedle delivered DNA vaccine promotes strong immunity [47,49,50]. We purchased the microneedle delivery system in 2015 and explored whether microneedle delivery would work for DNA vaccination in the rabbit model system. To overcome the complication of the slower growth of the hybrid genome, we chose another strong epitope DNA vaccine (CRPVE1ep1-5) which showed complete protection against the wild type CRPV infection in previous gene gun vaccination studies [38]. While we were planning the three group experiments, the gene gun system was encountering supplier changes and reagents would not be consistent with us did many years ago. We decided to omit the gene gun group of this comparative study. Twelve HLA-A2.1 transgenic rabbits were used for the current study. Three out of four rabbits vaccinated by both tattoo gun and microneedle were protected against CRPV infection during the entire study respectively. A significant difference between these two groups *vs.* the control group was found (supplementary Figure 1, Table 3, the fisher's exact test). These animals also generated epitope-specific tetramers (Table 3). Significantly smaller tumors were found in both tattoo gun, and microneedle vaccinated groups when compared with the control group (Figure 4, P<0.05 one-way ANOVA analysis). No significant difference was found 3 between microneedle and tattoo gun delivery groups (Figure 4, P>0.05 one-way ANOVA analysis).

DNA Vaccination by Tattoo Gun and Microneedle Stimulated Specific Killing to Epitope Pulsed Cells in HLA-A2.1 Transgenic Rabbits

Our previous studies have demonstrated that DNA vaccination stimulated epitope-specific CTL responses [39]. To test whether tattoo gun-mediated and microneedle-mediated DNA vaccinations stimulate epitope-specific CTL responses; we introduced a newly developed *in vivo* killing method to evaluate the specific-CTL events.

Table 4: Labeled and peptide (tested peptide CRPVE1/303-311 and a control peptide HIV gag/pol 17/77-85) pulsed autologous peripheral blood cells were retrieved 24 hours after infusion from the tested animals by *in vivo* killing assay.

Group	Rabbit	Vaccine	Test Peptide Pulsed PBLs (%)		Control Peptide Pulsed PBLs (%)		% Specific Killing
			Before	After	Before	After	
A	R4265	CRPVE1ep1-5	42.9	4	57.1	96	92
	R4276	DNA vaccine via	42	8	58	92	82
	R4284	tattoo gun	53.6	17.6	46.4	82.4	72
B	R4266	CRPVE1ep1-5	46	15	54	85	68
	R4269	DNA vaccine via	64.9	38	35.1	62	50
	R4286	microneedles	44	4	56	96	92
C	R4274	HPV16E7/82-90	35.7	30.5	54.3	69.5	9
	R4278	DNA vaccine via microneedles	50	39.6	50.5	60.4	9

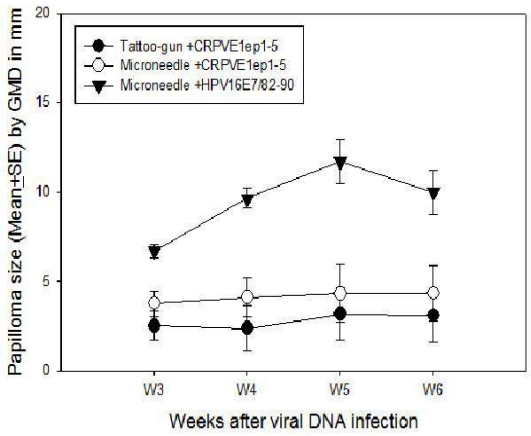

Figure 4: Tumor growth after viral infection in HLA-A2.1 transgenic rabbits [N=4/ group] vaccinated with CRPVE1ep1-5 DNA vaccine via tattoo gun or microneedle delivery method. A control group vaccinated with HPV16E7/82-90 via microneedle. After three vaccinations with a three-week interval, the animals were challenged with 5 μg wild type CRPV DNA. The tumor growth was monitored weekly and significantly smaller papillomas induced by both were found in vaccinated HLA-A2.1 transgenic rabbits when compared with those from the control epitope vaccinated animals at all the time points[$P<0.05$, one way ANOVA analysis]. No significant difference was found between the two delivery methods after DNA vaccination in HLA-A2.1 transgenic rabbits [$P>0.05$, one way ANOVA analysis].

One week after the final vaccination, rabbit PBLs were separated and pulsed with either a specific peptide (CRPVE1/303-311) or a control peptide (HIV gag/pol 17/77-85). The PBLs were subsequently labeled with different concentrations of CFSE and infused back into the animals. Twenty-four hours later, the labeled PBLs were retrieved from the blood and analyzed by flow cytometry. Animals from both tattoo and microneedle groups demonstrated several fold reduction in specific epitope-pulsed PBL populations, indicating that specific killing occurred in these animals. These data correlated with the protection (Table 4) and were an appropriate measure of the efficacy of immunization in animals.

Discussion

In this study, the CRPV/HLA-A2.1 transgenic rabbit model was used to assess the protective immunity generated by DNA vaccines delivered using the gene gun, the tattoo gun or the microneedle system. The focus was to determine whether the tattoo gun and the microneedle delivery systems were useful DNA vaccination alternatives to the gene gun. Our laboratory has successfully utilized the gene gun in our DNA vaccination studies for both protective and therapeutic purposes [17,34]. However, the gene gun system is costly, and we are constantly looking for alternatives to back up our vaccine development program. Tattoo gun has been reported to deliver a DNA vaccine successfully in both mice and non-human primates [21-23]. Compared with the gene gun and microneedle, tattoo gun delivery is more cost effective but more invasive which generates trauma and scars the vaccinated tissues. Microneedle delivery has achieved comparable protection in mice with twice the dose of gene gun delivery [24]. However, no studies have been attempted in rabbits for DNA vaccination with either a tattoo gun or a microneedle. In the current study, we compared the gene gun with the tattoo gun as well as the tattoo gun with microneedle DNA vaccine delivery using our well-studied DNA epitope vaccines and our unique HLA-A2.1 transgenic rabbit model [39]. Read out was tumor outgrowth rates and tumor size. We demonstrated that these two novel DNA delivery systems can be used for DNA vaccination in the rabbit model. The preparation and coding efficacy of bullets for gene gun delivery mainly depend on the quality of tubing and relevant reagents. We encountered discontinuation of tubing from the original suppliers, and the new supplier gave less satisfactory coating. Therefore, the dose for the gene gun delivery system may vary from each preparation. These inconsistencies and unavoidable variation drove us to find novel delivery methods for our DNA vaccination studies. In the last comparative experiments, we had to exclude the gene gun delivery method due to some uncontrollable variations in the tubing coating and other reagents that are critical for DNA coating and delivery. For tattoo gun and microneedle delivery, the DNA dose is much easier to monitor and control. At the time when we started the tattoo gun and the gene gun studies, the microneedle delivery was not available. Therefore, we compared the gene gun and tattoo gun in the first two experiments reported here. HPV16E7/82-90 DNA epitope vaccine has been demonstrated effective to protect against a hybrid CRPV genome containing HPV16E7/82-90 by gene gun delivery [39]. We achieved the similar levels of protection by tattoo gun vaccination in this study. The protection stimulated by tattoo gun vaccination protected the HLA-A2.1 transgenic rabbit not only against a hybrid CRPV infection but also a more vigorous tandem repeat (the CRPV/E7 (82-90)TR genome) used in our previous studies [hybrid genome plus half of the wildtype genome] [51]. Reported studies using tattoo gun delivered 100μg/ immunization to mice [21,28] and 650μg/ immunization to non-human primates [23], much higher doses than we used in the current studies. We achieved the efficacy comparable to that of gene gun delivery, indicating a lower dose could be just as effective for tattoo gun delivery. Collectively, these studies demonstrate

Supplementary Figure 1: Tumor growth at week 7 after viral infection in HLA-A2.1 transgenic rabbits [N=4/ group] vaccinated with CRPVE1ep1-5 DNA vaccine via tattoo gun [Group A] or microneedle [Group B] delivery method. A control group [Group C] vaccinated with HPV16E7/82-90 via microneedle. After three vaccinations with a three-week interval, the animals were challenged with 5 μg wild type CRPV DNA. Three and two out of four animals were free of tumors in the tattoo gun and microneedle vaccination group respectively. Two non-responders (R4276 and R4277) from group A and B respectively were from the same parents as R4278 from group C.

that the tattoo gun is a convenient and cost-effective alternative to the gene gun for DNA vaccination. However, the trauma created by the procedure is unfavorable for human use. We have been searching for a less invasive DNA delivery system to deliver DNA vaccine for many years. We acquired the microneedle delivery system several years following the first two studies. Microneedle system has shown promising results in animal studies [25,47,49]. The most appealing factor of microneedles delivery is that it induces minimal pain in human studies [24]. Therefore, it can be more acceptable for future human use. Microneedle applied DNA directly to the vaccinated sites; it is easier to control the delivery dose when compared with the gene gun that several preparation steps are involved to cause loss of DNA and consistency. In the third comparative study, we did not include a gene gun group because of unprecedented changes in resources of reagents for coating the bullets. Instead, we conducted a side-by-side comparative study using the microneedle and the tattoo gun vaccination. We applied the same amount of DNA vaccine that was used for gene gun delivery for both delivery methods. Our results demonstrated that microneedle was not only effective but also comparable to the tattoo gun delivery system. The protective immunity by both tattoo gun and microneedle delivered DNA vaccination was further confirmed with in vitro tetramer binding assay and *in vivo* CTL killing assay, indicating specific CTL production eliminated the infected cells in these immunized animals. As we have done for other studies with the in-house bred animals, we distributed the animals evenly among the groups to reduce the variation [32,52]. Interestingly, we observed one animal from each group developed large and persistent tumors [Supplementary figure 1]. This phenomenon was not surprising for us because we used the outbred animals in the study. Individual differences were anticipated as that in the human studies. We further investigated whether these non-responding animals shared unique genetic traits. Indeed, they were from the same breeding pair. However, we did not detect any difference in HLA-A2.1 expression levels, indicating other factors besides MHCI expression levels may play a role in the persistence of these non-responders. In summary, DNA vaccination via gene gun, tattoo gun, and microneedle all provided strong protective immunity in our HLA-A2.1 transgenic rabbits. We demonstrated that both tattoo gun and microneedle were promising alternatives for the gene gun delivery system.

Acknowledgement

This work was supported by the Public Health Service, National Cancer Institute Grant R01 441CA47622 from the National Institutes of Health and by the Jake Gittlen Memorial Golf Tournament.

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