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RESEARCH PAPER

Determination of Depth-Dependent Intradermal Immunogenicity of Adjuvanted Inactivated Polio Vaccine Delivered by Microinjections via Hollow Microneedles

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ABSTRACT

Purpose The aim of this study was to investigate the depth-dependent intradermal immunogenicity of inactivated polio vaccine (IPV) delivered by depth-controlled microinjections via hollow microneedles (HMN) and to investigate antibody response enhancing effects of IPV immunization adjuvanted with CpG oligodeoxynucleotide 1826 (CpG) or cholera toxin (CT).

Methods A novel applicator for HMN was designed to permit depth- and volume-controlled microinjections. The applicator was used to immunize rats intradermally with monovalent IPV serotype 1 (IPV1) at injection depths ranging from 50 to 550 µm, or at 400 µm for CpG and CT adjuvanted immunization, which were compared to intramuscular immunization.

Results The applicator allowed accurate microinjections into rat skin at predetermined injection depths (50–900 µm), -volumes (1–100 µL) and -rates (up to 60 µL/min) with minimal volume loss (±1–2%). HMN-mediated intradermal immunization resulted in similar IgG and virus-neutralizing antibody titers as conventional intramuscular immunization. No differences in IgG titers were observed as function of injection depth, however IgG titers were significantly increased in the CpG and CT adjuvanted groups (7-fold).

Conclusion Intradermal immunogenicity of IPV1 was not affected by injection depth. CpG and CT were potent adjuvants for both intradermal and intramuscular immunization, allowing effective vaccination upon a minimally-invasive single intradermal microinjection by HMN.

ABBREVIATIONS

CpG	CpG oligodeoxynucleotide 1826
CT	Cholera toxin
DC	Dendritic cell
DDC	Dermal dendritic cell
H&E	Hematoxylin and eosin
HMN	Hollow microneedle
IPV	Inactivated polio vaccine
IPV1	Monovalent inactivated polio vaccine serotype 1
LC	Langerhans cell
OPV	Oral polio vaccine
PBS	Phosphate buffered saline
TMB	$3,3'5,5'$-tetramethylbenzidine
VN	Virus neutralizing

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INTRODUCTION

Poliomyelitis can be prevented through vaccination by either oral polio vaccine (OPV) or inactivated polio vaccine (IPV). Although OPV is an inexpensive and easy to administer vaccine, it may cause outbreaks of vaccine-derived polioviruses (1). Therefore, the World Health Organization aims to eliminate the use of OPV and substitute it by IPV in its goal towards worldwide polio eradication (2). However, IPV vaccination is more costly because of higher production costs and a higher dose requirement in comparison to OPV. Therefore,

several strategies for dose sparing to reduce the cost of IPV vaccination have been proposed, including intradermal immunization and IPV adjuvantation (3).

Although intradermal IPV immunization at reduced doses in human resulted in seroconversion (4), intradermal injection by the Mantoux technique is difficult to perform. Therefore, there is an urgent need for novel intradermal injection methods. Intradermal IPV immunization via jet injectors resulted in seroconversion at reduced doses (5,6). Another novel strategy for intradermal injection is microneedles, which are micron-sized needles (7). Microneedles were investigated on their ability to induce protective immune responses upon intradermal IPV delivery on rats and rhesus macaques (8–12). Hollow microneedle (HMN) mediated intradermal IPV immunization at reduced doses was also investigated in humans, which resulted in similar seroconversion rates in comparison to a full intramuscular dose (13).

Although intradermal immunization is a promising immunization strategy, it is unknown whether the immunogenicity of intradermally administered IPV is affected as a function of injection depth. This is of interest, as several classes of dendritic cells (DCs) reside in either the epidermis or the dermis. Langerhans cells (LCs) reside in the epidermis (topmost skin layer) and dermal dendritic cells (DDCs) reside in the dermis (lower layer) (14). Both LCs and DDCs have distinct immune functions and may therefore have a different role in immunization (14). Therefore, targeting different skin depths may affect the efficiency of intradermal IPV immunization. Hence, the objective of the present study is to investigate intradermal IPV immunization efficiency as function of skin injection depth by using a HMN system. To this end, precise and reproducible injection of IPV into the skin at a predefined depth is a requirement. This requirement can be fulfilled by using our previously developed in-house applicator (8). However, this applicator only allowed a maximum flow rate of 2 μL/min without leakage in the microinjection system. Therefore, the system was thoroughly redesigned to allow increased pressures, resulting in increased ranges of injection rates, −volumes and -depths without leakages.

Besides establishing the potential effect of intradermal injection depth on the immune response, the use of adjuvants can improve IPV immunization efficiency and therefore may result in IPV dose sparing and thus cost reduction (3,15). Although colloidal aluminum hydroxide or aluminum phosphate salts have been used with IPV for intramuscular immunization (16–20), they are not for intradermal use as they cause severe side effects. Therefore, in this study we examined the immune potentiating effects of CpG oligodeoxynucleotide 1826 (CpG) and cholera toxin (CT) as potentially suitable adjuvants for intradermal IPV immunization.

In this study we demonstrate a HMN applicator that allows for injection depth, -rate and -volume controllable minimally-invasive intradermal microinjections. This applicator allowed to investigate the dependence of intradermal immunogenicity to the injection depth of IPV1. To our knowledge this is the first systematic study where the influence of injection depth on antigen-specific immune responses is investigated. Additionally, the potential of adjuvants CpG and CT to enhance intradermal IPV immunization for dose sparing was investigated.

MATERIALS AND METHODS

Materials

Polyimide coated fused silica capillary (375 μm outer diameter, 100 and 20 μm inner diameter) was obtained from Polymicro, Phoenix, USA. Silicone oil AK 350 was purchased from Boom Chemicals, Meppel, the Netherlands. CapTite™ connections were obtained from Labsmith, USA. Parafilm was purchased from Bemis, Monceau-sur-Sambre, Belgium. Tissue-Tek O.C.T. compound was ordered at Sakura Finetek, Alphen aan den Rijn, the Netherlands. Phosphate buffered saline, pH 7.4 (PBS pH 7.4: 163.9 mM Na^+, 140.3 mM Cl^-, 8.7 mM HPO_4^{2-} and 1.8 mM $H_2PO_4^-$, pH 7.4) was purchased from B. Braun Melsungen, Melsungen, Germany. IsoFlo® (isoflurane 100% w/w) was obtained from Abbott Laboratories, Maidenhead, UK. CpG was purchased from Invivogen, Toulouse, France. Hydrofluoric acid (49% w/w), concentrated sulfuric acid (95–98%), fluorescein, trypan blue, CT (holotoxin) and 3,3′5,5′-tetramethylbenzidine (TMB) were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands. Polystyrene 96 well microtiter plates and 2.5 mL Vacuette® Z serum separator clot activator premium tubes were ordered at Greiner Bio-One, Alphen aan den Rijn, the Netherlands. Tween 80 and 30% w/w hydrogen peroxide were obtained from Merck, Amsterdam, the Netherlands. PBS pH 7.2 (160.6 mM Na^+, 155.2 mM Cl^-, 2.7 mM HPO_4^{2-}, 1.5 mM $H_2PO_4^-$ and 1.5 mM K^+, pH 7.2) was purchased from Gibco (Life Technologies), Bleiswijk, the Netherlands. Protifar was obtained from Nutricia, Zoetermeer, the Netherlands. Bovine anti-poliovirus type 1 serum, monovalent IPV vaccine serotype 1 (IPV1), 1.1 M sodium acetate and 2 M sulfuric acid were kindly provided by Intravacc, Bilthoven, the Netherlands. Horseradish peroxidase-conjugated goat-anti-rat IgG was obtained from Southern Biotech, Birmingham, AL, USA. Female Wistar Han IGS rats (Crl:WI(Han), strain code 273) of 175–225 g were ordered from Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France.

Fabrication of HMN

HMN were produced by an in-house process as described previously (8). In short, the inner lumen of 20 μm inner

diameter polyimide coated fused silica capillaries were filled overnight with silicone oil AK 350 by use of a vacuum oven at 100°C. These silicone oil-filled capillaries were subsequently wet etched into HMN by immersing the ends in a container with hydrofluoric acid (49% w/w) for 4 h. To expose the microneedle tips, the polyimide coating at the etched ends of the capillaries was removed by immersing them in concentrated sulfuric acid (95–98%) at 250°C for 5 min.

HMN Applicator

The HMN applicator that was previously developed in-house (8) was thoroughly redesigned to enable better depth-controlled intradermal microinjections. This HMN applicator is depicted in Fig. 1a and b. Optimization was dedicated to improve accuracy, precision and reproducibility of microinjections, to allow for increased injection rates to decrease the injection time, to allow for higher injection volumes and to achieve skin depth-controlled injections. To achieve these improvements, high-pressure resistance in the fluidics system was required. Therefore, flexible materials were replaced by nonflexible rigid materials and all previous connectors in the fluidics system were replaced by high-pressure resistant CapTite™ connectors. For the fluidics system, an 100 µL Hamilton gas-tight Luer-Lock syringe (model 1710 TLL, Hamilton Robotics, Bonaduz, Switzerland) with a barrel inner diameter of 1.46 mm was used in conjunction with a syringe pump (NE-300, Prosense, Oosterhout, the Netherlands) and 100 µm inner diameter polyimide coated fused silica capillaries.

HMN Applicator Performance Validation with *Ex-Vivo* Rat Skin

Microinjections into *ex-vivo* rat skin were performed to determine the range of injection rates, −volumes and –depths that can be used for leakage-free microinjections. *Ex-vivo* shaved rat skin was isolated from sacrificed female Wistar Han rats. The skin was stretched on Styrofoam covered with parafilm. Subsequently, microinjections of a solution of 10 µg/mL fluorescein in PBS pH 7.4 were performed at various injection rates, −volumes and -depths. To determine the accuracy and repeatability of the microinjections, volume loss that occurred at i) connections of the fluidics system, ii) on the skin surface at the injection site or iii) due to retained volume on the microneedle after its withdrawal from the injection site was measured by pipetting the lost volume with a 0.2–2 µL pipette. A pipetted volume below 0.2 µL was measured as a volume loss of 0.2 µL. The percentage volume loss was calculated as the percentage of volume loss (as measured by pipetting) from the digitally-displayed dispensed volume (as indicated by the syringe pump).

In the first series of experiments, the injection rate performance was investigated by varying injection rates from 1 to 60 µL/min, while both the injection volume and -depth were kept constant at 10 µL and at 500 µm, respectively. The maximum injection rate of the syringe pump for the 1.46 mm inner diameter 100 µL Hamilton syringe was 62 µL/min. In the second series of experiments, the injection volume was investigated by varying injection volumes from 1 to 100 µL, while both the injection rate and -depth were kept constant at 20 µL/min and at 500 µm, respectively. Finally, injection depths ranging from 50 to 900 µm were investigated by performing 10 µL microinjections at an injection rate of 20 µL/min.

Visualization of Depth-Controlled Microinjections in *Ex-Vivo* Rat Skin

In order to visualize microinjections at different preselected depths in *ex-vivo* rat skin, trypan blue solution (0.4% w/v in PBS pH 7.4) was used. *Ex-vivo* rat skin was isolated and prepared as described above. Next, trypan blue solution was injected at 250, 400 and 550 µm depths with an injection volume of 10 µL and at an injection rate of 20 µL/min. The microinjections in *ex-vivo* rat skin were photographed on outer and inner skin sides by utilizing a stereo microscope at 2.5× magnification (Zeiss Stemi 2000-c, paired with a Zeiss AxioCam ICc5 camera).

Furthermore, *ex-vivo* rat skin injected with 0.5 µL trypan blue solution at the same injection depths and at an injection rate of 1 µL/min was embedded in Tissue-Tek O.C.T. compound and subsequently snap-frozen in liquid nitrogen, from which 10 µm thick cryosections were made on a Leica CM3050s cryostat. Subsequently, unstained and hematoxylin and eosin (H&E) stained images were made to visualize the injection depth of the microinjections. Images were made with a Zeiss 10× plan-Apochromat objective mounted onto a Zeiss Axio Imager D2 microscope coupled with a MRc5 camera.

Investigation of Depth-Dependent Intradermal Immunogenicity

Two immunization studies were performed under the guidelines and regulations enforced by the animal ethic committee of the Netherlands, and were approved by the "Dierexperimentencommissie Universiteit Leiden (UDEC)" under number 12084. Female Wistar Han rats with a weight of 175–225 g on arrival were accommodated under standardized conditions in the animal facilities of the Leiden Academic Centre for Drug Research, Leiden University. The animals were housed in groups of 5 and were assigned to different immunization groups (10 rats per immunization group). Prior to blood withdrawal or immunization, the rats were anaesthetized with isoflurane. Anaesthetized animals that

were assigned to the intradermal injection groups were shaved minimally (an area of 4 cm^2 on the left flank) prior to the intradermal injection.

To investigate the depth-dependent intradermal immunogenicity of IPV1, the rats were immunized intradermally via HMN mediated microinjections of 10 µL containing 5 DU of IPV1 at an injection rate of 20 µL/min and at injection depths of 250, 400, 550 µm for animal study 1, and 50, 150, 250 µm for animal study 2. As a control in both animal studies, 5 DU of IPV1 in 200 µL PBS pH 7.4 was administered intramuscularly divided over each hind leg (100 µL per hind leg). Furthermore, both animal studies contained a mock treated group via the intramuscular route (100 µL PBS pH 7.4 per hind leg). In the first animal study the immunogenicity enhancing effects of adjuvants were investigated: two groups were immunized intradermally via HMN mediated microinjections of 10 µL (injection rate 20 µL/min and injection depth 400 µm) and two groups were immunized intramuscularly (100 µL per hind leg) with 5 DU IPV1 adjuvanted with either CpG or CT. These previously described immunization procedures were performed at day 1 (prime immunization) and were repeated at day 21 (booster immunization). Collection of blood samples were performed at day 1, day 21 (prime) and day 42 (boost). Blood samples were collected in 2.5 mL Vacuette® tubes and stored on ice before centrifugation at 2000 g for 10 min to isolate serum.

Serum IgG Titers

To measure serum IgG titers, a capture ELISA was performed. Bovine anti-poliovirus type 1 serum in PBS pH 7.2 was used to coat polystyrene 96 well microtiter plates overnight at 4°C. Subsequently, washing was performed with 0.05% Tween 80 in tap water. Afterwards, assay buffer consisting of PBS pH 7.2, 0.5% (w/v) Protifar and 0.05% (v/v) Tween 80 was used to add 4.5 DU IPV1/well (100 µL/well). Plates were incubated at 37°C for 2 h before a wash step was performed. Afterwards, threefold serial dilutions of serum samples in assay buffer were added at 100 µL/ well and subsequently incubated at 37°C for 2 h. Plates were washed before horseradish peroxidase-conjugated goat-anti-rat IgG was added to the wells (4000-fold dilution, 100 µL/ well) and subsequently incubated at 37°C for 1 h. Subsequently, plates were thoroughly washed before adding TMB substrate solution (100 µL/well) which consisted of 1.1 M sodium acetate, 100 mg/mL TMB and 0.006% (v/v) hydrogen peroxide. 2 M sulfuric acid was used after 10 min to stop the reaction (100 µL/well). Finally, sample absorbance was measured at 450 nm by a Biotek ELx808 plate reader (Winooski, VT, USA).

The Biotek Gen5 2.0 data analysis software was used to determine endpoint titers by 4-parameter analysis. The endpoint titer was defined as the reciprocal of the serum dilution producing a 450 nm absorbance equal to that of the mean 450 nm absorbance with addition of three times the standard deviation of eight samples of IPV1-specific-antibody negative serum samples.

Virus-Neutralizing Antibodies

Determination of the antibody titers able to neutralize wildtype poliovirus serotype 1 was outsourced to Bilthoven Biologicals and performed as previously described (21,22). In short, average virus-neutralizing (VN) antibody titers were measured after pooling of the serum samples of all rats per immunization group. Subsequently, two-fold serial dilutions of the pooled sera were made (2^{11}–2^{22}) after inactivation of sera at 56°C for 30 min prior to testing. 100TCID$_{50}$ of the Mahoney wild-type strain (poliovirus type 1) was added to the resulting serum dilutions and these virus/serum mixtures were incubated for 3 h at 36°C and 5% CO$_2$ before incubation at 4°C overnight. Subsequently, to each sample 1×10^4 Vero cells were added and the resulting mixtures were incubated for 7 days at 36°C and 5% CO$_2$. Finally, samples were fixed with formalin, stained with crystal violet and were analyzed macroscopically. VN titers were displayed as the last serum dilution which did not exhibit cytopathogenic effects.

Statistical Analysis

Statistics were performed using GraphPad Prism (v.6.00, GraphPad Software, LaJolla, CA, USA). Kruskall-Wallis tests with Dunn's post-hoc tests were performed as IgG titers were non-normally distributed and considered significant at $p < 0.05$.

RESULTS

HMN Applicator Optimization and Performance

To allow for increased injection rates and thereby short injection times, connections in the fluidics system of the HMN applicator were optimized by applying high-pressure resistant CapTite™ connectors and high-pressure resistant polyimide fused silica capillaries, as shown in Fig. 1a and b. Furthermore, the dead volume in the fluidics system was kept to a minimum to maximize injection volume accuracy and minimize the loss of vaccine formulation. The hydrofluoric acid etching procedure of polyimide coated fused silica capillaries resulted in sharp HMN (Fig. 1c).

The performance of the fluidics system was examined by varying the injection rate during intradermal microinjections into *ex-vivo* rat skin, mimicking back pressure during actual microinjections on live animals. As shown in Fig. 2a, the volume loss was ±1–2% for all investigated injection rates at an
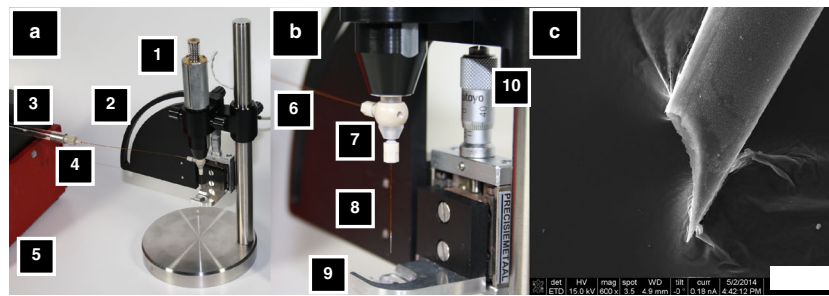
Fig. 1 Images of the HMN applicator (**a** and **b**) and a scanning electron microscopy image of a HMN (**c**, bar represents 50 μm). The microneedle insertion speed (1 to 3 m/s) is controlled by an electromagnet (1). Angled injections are possible via a guided rail (2). The injection depth of the microneedle into the skin is accurately controlled by a micrometer actuator (10) and a guide plate (9). Fluid flow starts with a 100 μL Hamilton gas-tight Luer-Lock syringe (3) which is driven by a controllable syringe pump (5) that is connected to a 100 μm inner diameter capillary (6) via a Luer-Lock-CapTite adapter (4). This capillary feeds the fluid flow via a specially designed connection piece (7) into a HMN (8) that enables the intradermal injections. Because the syringe pump is programmable, injection rates and -volumes can be accurately controlled.

injection volume and -depth of 10 μL and 500 μm, respectively. Volume loss was only observed at the skin surface of the microinjection sites or on the HMN after retracting the microneedle from the skin. No additional volume loss was observed. Owing to the achieved increase in injection rate, a significant reduction in injection time was achieved.

As shown in Fig. 2b, volumes up to 100 μL could be injected into *ex-vivo* rat skin with volume loss that was ±1–2%, independent of injection volume, at an injection depth and -rate of 500 μm and 20 μL/min, respectively. This volume loss was observed on the skin surface at the microinjection sites.

Finally, variation in injection depth was studied by injecting 10 μL in *ex-vivo* rat skin at different injection depths. As shown in Fig. 2c, microinjections were performed between 50 and 900 μm injection depths. At all investigated injection depths, all microinjections resulted in ±1–2% volume loss. Moreover, there was no increase in volume loss at any particular injection depth.

## Visualization of Depth-Controlled Microinjections in *Ex-Vivo* Rat Skin

To obtain evidence that skin layers at different preselected depths were targeted, microinjections of a trypan blue solution were performed on *ex-vivo* rat skin, which was then photographed at both sides (Fig. 3a). Whereas the trypan blue spot of the shallower microinjection at a skin injection depth of 250 μm was more clearly visible at the outer side of the skin, the trypan blue spot of the deeper injection at 550 μm was hardly visible. Contrarily, the trypan blue spot of the deeper microinjection at a skin injection depth of 550 μm was more clearly visible at the inner side of the skin than the trypan blue spot of the shallower injection (250 μm). Moreover, as shown in Fig. 3b, visualization of the skin injection depth in cross-sectioned skin indicated that the microinjections were indeed performed at different pre-defined skin injection depths.

Injection Depth-Dependent Intradermal Immunogenicity

To assess whether injection of IPV1 at different injection depths in the skin affects IPV1-specific antibody responses, two immunization studies in rats were conducted. Intradermal immunization was performed using the HMN applicator in a depth-controlled manner and this was compared to intramuscular immunization with a conventional 26G hypodermic needle. IPV1-specific IgG titers obtained after intradermal immunization with non-adjuvanted IPV1 at injection depths ranging between 50 and 550 μm are shown in Fig. 4. Three weeks after prime immunization (Fig. 4a) and boost immunization (Fig. 4b), no significant differences in IPV1-specific IgG titers were observed at different injection depths. Moreover, IPV1-specific IgG titers obtained after intradermal immunization were similar to those obtained after intramuscular IPV1 immunization. No IPV1-specific antibody responses were observed in the mock treated group.

Immunogenicity Enhancing Effects of Adjuvants CpG and CT

To assess the potential increase in antibody responses and the applicability for use as intradermal adjuvants, CpG and CT were used as adjuvants in intradermal and intramuscular IPV immunization (Fig. 4a and b). Intradermal IPV1 immunization adjuvanted with CpG led to statistically significant increased IgG titers in comparison to non-adjuvanted 400 and 550 μm injection depths and intramuscular IPV1 group after prime immunization. Contrarily, intramuscular IPV1 immunization adjuvanted with CpG did not result in any significant increase in IgG titer after prime immunization. After boost immunization however, IPV1 immunization adjuvanted with CpG via both the intradermal and intramuscular route resulted in significantly increased IgG titers in comparison to the non-adjuvanted intramuscular IPV1 group.
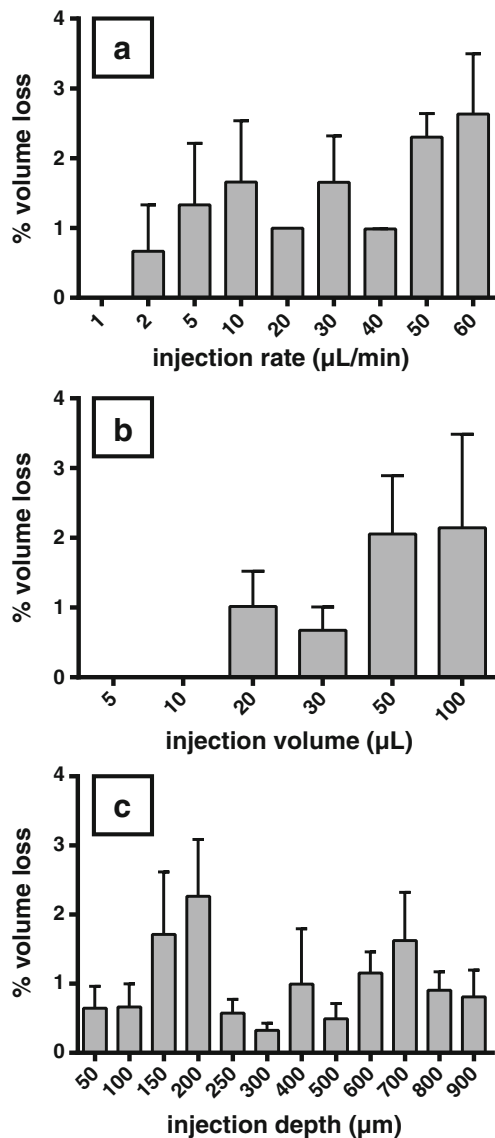
Fig. 2 Accuracy of the HMN applicator, expressed as percent volume loss, as function of (**a**) injection rate (1–60 μL/min (pump-syringe combination maximum)) at constant injection depth (500 μm) and volume (10 μL); (**b**) injection volume (5–100 μL) at constant injection depth (500 μm) and rate (20 μL/min); and (**c**) injection depth (50–900 μm) at constant injection volume (10 μL) and rate (20 μL/min). *Bars* represent mean ± SEM ($n = 6$).

Intradermal IPV1 immunization adjuvanted with CT led to statistically significant increased IgG titers after prime immunization (non-adjuvanted 400 µm injection depth) and booster immunization (non-adjuvanted intramuscular control group). Intramuscular IPV1 immunization adjuvanted with CT resulted in statistically significant increased IgG titers in comparison to all non-adjuvanted groups (250, 400 and 550 µm injection depths and intramuscular IPV1 group) after prime immunization. However, after boost immunization there were no significant differences for intramuscular IPV1 immunization adjuvanted with CT.

Furthermore, there were no statistically significant differences in IgG titers between CpG and CT adjuvanted groups, neither for the intradermal or the intramuscular immunization routes and neither after prime or boost immunization. However, some differences were observed between CpG and CT, when they were compared against non-adjuvanted groups. After prime intradermal immunization, CpG and CT adjuvanted IPV1 immunization resulted in 6.9 and 7.3 fold increased IgG titers on average, respectively, in comparison to the non-adjuvanted intradermal IPV1 groups. After prime intramuscular immunization, CpG and CT adjuvanted IPV1 immunization resulted in 2.7 and 12.1 fold increased IgG titers, respectively, in comparison to the non-adjuvanted intramuscular IPV1 group. Thus in prime immunization, CpG enhanced IgG titers more in intradermal immunization in comparison to intramuscular immunization, whereas CT enhanced IgG titers in both immunization routes. After the booster intradermal immunization, CpG and CT adjuvanted IPV1 immunization resulted in 1.9 and 2.2 fold increased IgG titers on average, respectively, in comparison to the non-adjuvanted intradermal IPV1 groups. After the booster intramuscular immunization, CpG and CT adjuvanted IPV1 immunization resulted in 5.5 and 2.4 fold increased IgG titers, respectively, in comparison to the non-adjuvanted intramuscular IPV1 group. Thus, the IgG titer enhancing effect was more pronounced in prime immunization than in booster immunization. Intramuscular IPV1 immunization adjuvanted with CpG resulted in the strongest increase in IgG titers in booster immunization.

Besides increasing IgG titers, intradermal IPV1 immunization adjuvanted with CpG and both intradermal and intramuscular IPV1 immunization adjuvanted with CT, resulted in less low-responders in comparison to the non-adjuvanted groups after prime immunization. Moreover, these adjuvanted groups resulted in IPV1-specific IgG titers after a single immunization that were comparable to the ones obtained after two non-adjuvanted immunizations.

Virus-Neutralizing Antibodies

In addition to the determination of the IPV1-specific IgG antibody responses by ELISA, protectivity of these antibodies against wildtype poliovirus was measured in a wildtype poliovirus-neutralizing antibody assay. VN antibody titers were observed in all IPV1 immunization groups (Table I). No VN antibody titer was observed in the mock treated group. Although the VN titers were measured on pooled serum samples, some slight differences in mean VN titer per immunization group were observed. For example, for the

Fig. 3 Visualization of depth-controlled microinjections of trypan blue solution by HMN into rat skin at 10 µL and 20 µL/min (**a**), visualized by light microscopy at 4× magnification; *bars* represent 500 µm. Visualization of cross-sections of cryofixed rat skin after depth-controlled microinjections of trypan blue solution into rat skin at 0.5 µL at 10 µL/min were performed (**b**), visualized either before or after H&E staining by bright-field microscopy at 10× magnification; *bars* represent 200 µm.
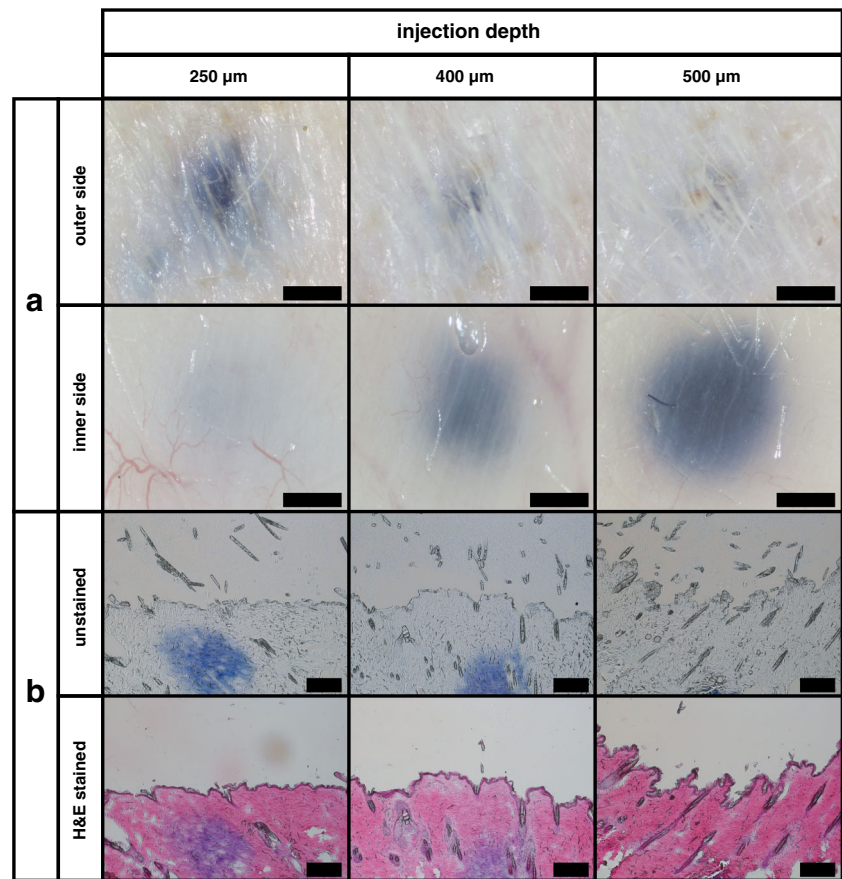

400 µm injection depth, a VN titer of 13 was observed, which was higher than those of the other injection depths and the intramuscular IPV1 immunization. Moreover, this VN titer was similar to the VN titers of intradermal and intramuscular IPV1 immunization adjuvanted with CpG, however lower than the VN titer of intradermal IPV1 immunization adjuvanted with CT, which had the highest VN titer. VN titers of non-adjuvanted intradermal and intramuscular IPV1 immunization were similar. Intramuscular IPV1 immunization adjuvanted with CT resulted in a similar VN titer as non-adjuvanted intradermal and intramuscular immunization and was lower in comparison to intradermal IPV1 immunization adjuvanted with CT. Thus, CpG enhanced VN antibody titers in both intradermal and intramuscular IPV1 immunization, whereas CT only in intradermal IPV1 immunization.

Both during and immediately after intradermal immunization with HMN, a small bleb was observed on the skin. This bleb disappeared within 5 min after injection. No erythema was observed during and after intradermal injections with HMN, with one exception. Mild erythema was observed after intradermal injection of IPV1 with CT. This effect was only observed after the prime immunization and started 60 min post injection and lasted up to 7 days. Other adverse effects were not observed.

DISCUSSION

The relationship between injection depth and intradermal immunization has not been studied before, because this requires accurate, precise and reproducible depth-controlled intradermal injections with small injection volumes. For these reasons, an HMN applicator was thoroughly redesigned and improved in this study. This improved HMN applicator allowed for accurately controllable injection rates, -volumes and -depths, which allowed to investigate the depth-dependent intradermal immunogenicity of IPV1, in an effort for IPV1 dose sparing.

Skin resistance during injection might significantly inhibit injection rate and accuracy (23). However, the redesigned applicator allowed for a 30-fold increased injection rate (60 µL/min) compared to previously achieved rates (2 µL/min) (8). Increased injection rates were needed to increase injection accuracy and shorten injection duration. Achieved injection rates were higher than those reported in literature for rat skin with a single HMN (0.17 µL/min/HMN, 1 µL/min/HMN after needle retraction, (24)) or with HMN arrays (0.001 µL/min/HMN, (25)) and for human skin with a single HMN (0.25–1.6 µL/min/HMN), which required hyaluronidase and needle retraction to increase rates to 18.83 µL/min/HMN (23). In contrast, by using the improved applicator, no
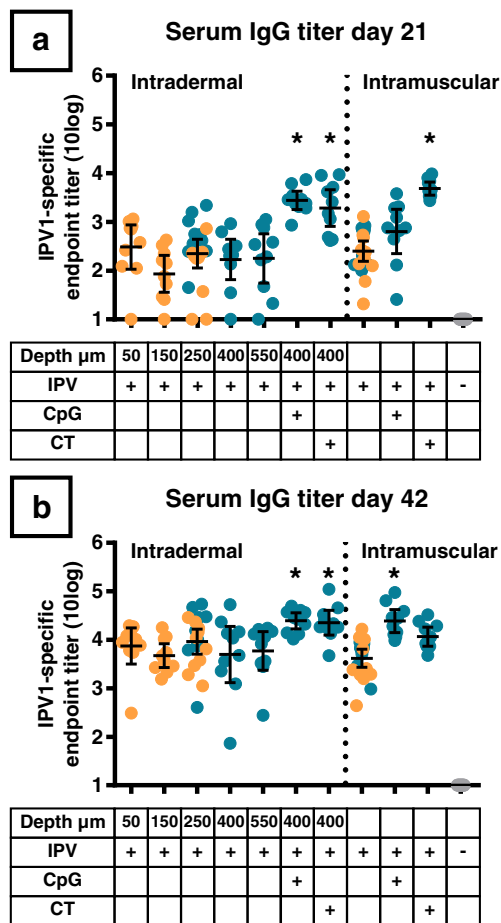
Fig. 4 IPV1-specific IgG antibody responses measured in serum obtained in study 1 or 2, after prime immunization (**a**, day 21) or boost immunization (**b**, day 42) with PBS pH 7.4, 5 DU IPV1 or 5 DU IPV1 adjuvanted with either CpG or CT. Formulations were injected intradermally *via* HMN at the indicated injection depths or intramuscularly *via* conventional 26G needles. *Aqua* or *orange* rounds represent animals from study 1 or 2, respectively. *Bars* represent mean ± 95% *confidence interval* (n = 10) and *stars* represent a significant increase (p < 0.05).

Table I Virus Neutralizing (VN) Antibody Titers Measured in Serum Obtained in Study 1 and 2 After Prime and Boost Immunization (Day 42) with PBS pH 7.4, 5 DU IPV1 or 5 DU IPV1 Adjuvanted with Either CpG or CT. Formulations Were Injected Intradermally *via* HMN at Indicated Injection Depths or Intramuscularly *via* Conventional 26G Needles. Presented Data Represents VN Titers Which Were Measured on Pooled Serum Samples of All Rats Per Immunization Group

VN antibody titers (log$_2$)

	Intradermal							Intramuscular			
Depth (µm)	50	150	250	400	550	400	400				
IPV1	+	+	+	+	+	+	+	+	+	+	−
CpG						+			+		
CT							+			+	
VN titer study 1			11	13	11	13	14	12	13	11	0
VN titer study 2	11	12	12					11			0

additional methods were required to achieve even higher injection rates. Therefore, the utilization of non-flexible materials allowed increased pressure during injection, which allowed to easily overcome skin resistance and to achieve increased injection rates.

In addition, injection volumes achieved with this improved applicator were increased 11-fold in comparison to those previously achieved in rat skin (9 µL/HMN) (8). These volumes were also much higher as those reported for single HMN in human skin (20 µL/HMN) (23) or HMN arrays in mice skin (0.22 µL/HMN) (26). Additionally, achieved volumes were comparable to HMN arrays in porcine skin (83.33 µL/HMN) (27). Furthermore, a single hollow needle design reduces the risk of volume loss (dose loss) compared to HMN arrays, as in arrays all needles need to be penetrated and unclogged to ensure an accurate injection and prevent leakage (28,29).

The improved applicator allowed for an increased injection depth range (50–900 µm) compared to the previously reported range (100–400 µm) (8). This injection depth range was larger than the previously reported depth range in hairless rat skin (150–770 µm, (24)) and smaller than the previously reported depth range in human skin (180–1080 µm, (23)). However, these studies do not report the influence of injection depth on antigen-specific immune responses.

In contrast to our hypothesis, there was no injection depth-dependent intradermal immunogenicity observed for IPV1. However, the limited thickness (20–30 µm) of rat epidermis prevented injection into the epidermis where LCs reside (30). The attempt to inject IPV1 as close to the epidermis as possible did not result in a significant change in IPV1-specific IgG titers. It is not known whether the distribution of the injected volume in the skin influences the targeting of intradermal injection depths. Furthermore, it is unclear whether local changes in innate immunity can be expected, as antigen-bearing DCs will migrate to lymph nodes, where lymph node-residing DCs may influence any immunological effect (14,30). This behavior, chemotaxis and chemoattraction of other DCs and the complex interplay between different DC classes, may explain the absence of differences in immune response as function of the injection depth. Contrarily to rat skin though, in human skin the epidermis has a thickness of 50–100 µm (30). As the redesigned applicator is able to inject at a depth of 50 µm, injection directly into the human epidermis is possible. This allows to study LC function in intradermal immunity in the future. In conclusion, our applicator could provide a method to investigate depth-dependent intradermal vaccine immunogenicity in human.

Furthermore, results presented in this study are important in microneedle patch design for intradermal IPV immunization as the independence of IPV1 immunization on skin depth suggests that microneedle length will not affect the immune response and thus precise dosing at a certain skin depth is not

required. This may put a smaller burden on the design specifications of microneedle applicators.

Besides, an effort was made to find a dose sparing strategy for IPV immunization. However, intradermal and intramuscular immunization without use of adjuvants led to similar results, likewise as reported for other microneedle strategies: HMN in rats (8,11) or humans (13), coated microneedles in rats (9), dissolving microneedles in rats (10) or rhesus macaques (12). Furthermore, clinical trials in humans have been performed to investigate IPV dose sparing, by comparison of a 80% reduced IPV dose (20% of full dose) administered intradermally against a full IPV dose administered intramuscularly. For polio prime immunization in newborn infants, intradermal 20% IPV dose resulted in similar (4,5) or inferior (31–33) seroconversion rates compared to a full intramuscular dose. Similarly, for polio booster immunization, intradermal 20% IPV dose resulted in either similar (6,13) or inferior (34,35) seroconversion rates in comparison to full intramuscular dose. However, intradermal immunization generally resulted in significantly lowered antibody titers (5,6,31–35). These findings were seemingly not dependent on the intradermal immunization method used, because jet injectors (5,6,31,32,34,35), the Mantoux technique (4,35) or HMN arrays (13,33) evenly resulted in either similar or inferior results to full intramuscular dose. In this study, it is shown that the depth at which the antigen was administered by these different intradermal immunization techniques cannot explain this phenomenon either. However, the 80% dose-reduction goal may be unrealistic, as a 60% dose-reduction was achieved by HMN mediated intradermal immunization without use of adjuvants (11,13).

Adjuvants may be used as another strategy for IPV dose sparing (3). For example, aluminum hydroxide (16,17) and aluminum phosphate (18,19) have been successfully used as an adjuvant for intramuscular IPV immunization. Because aluminum salts cannot be administered intradermally due to local adverse effects, adjuvants CpG and CT were assessed for intradermal IPV1 immunization. Both CpG and CT have shown to be safe as adjuvant in intradermal immunization in humans (36,37). In the present study, immune-enhancing effects on IPV1-specific IgG responses by the adjuvants CpG and CT were significant, which indicates that CpG and CT might be potential adjuvant candidates for intradermal IPV1 immunization and may lead to dose sparing. Indeed, CpG was reported by others to be an effective immune enhancing adjuvant for IPV immunization (38). Although this latter study utilized mice and a different CpG motif (ODN2006), both studies report on the potential for CpG as adjuvant in IPV immunization. Furthermore, we have reported a 6.9- and 7.3-fold increase in IPV1-specific IgG serum titers for intradermally IPV co-administered with CpG and CT, respectively. This increase was larger as reported for intradermal IPV immunization in mice in combination with CpG-ODN2006 (4-fold) or double mutant heat-labile enterotoxin from E. coli LT (R192G/L211A) (2.5-fold) (38,39). Contrarily, a 10-fold increase was achieved by CAF01 liposomal IPV formulation for intradermal IPV immunization in mice (40).

CONCLUSION

In this study, an unique dose sparing strategy for IPV1 was investigated by intradermal microinjections of small volumes of IPV1 formulation at different pre-determined depths in rat skin *in vivo*. To enable this, a HMN applicator controllable in injection rate, −volume and −depth was developed that allowed for intradermal microinjections. Results indicated however, that intradermal immunogenicity was not dependent on the injection depth. Nonetheless, IPV1 immunization by minimally-invasive intradermal microinjections resulted in similar IPV1-specific antibody responses in comparison to IPV1 immunization by more invasive and painful intramuscular injections. Moreover, intradermal IPV1 immunization antibody responses were significantly increased (7-fold) by adjuvants CpG and CT, such that a single minimally-invasive intradermal immunization by HMN resulted in comparable antibody responses to two intramuscular immunizations.

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