Novel formulations and delivery strategies for inactivated polio vaccines : new routes with benefits

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Alternative delivery of a thermostable inactivated polio vaccine

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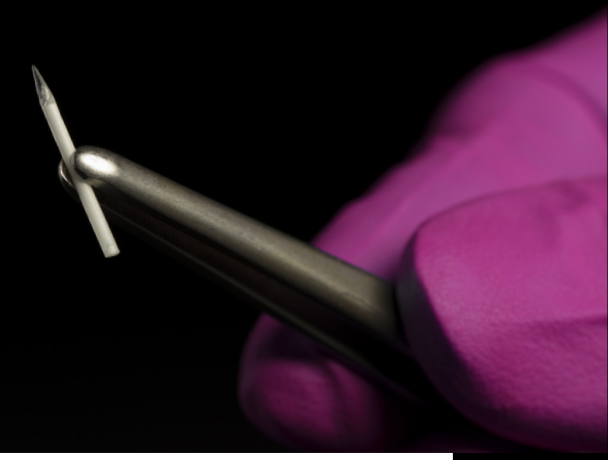

ABSTRACT

In the near future oral polio vaccine (OPV) will be replaced by inactivated polio vaccine (IPV) as part of the eradication program of polio. For that reason, there is a need for substantial amount of safe and more affordable IPV for low-income countries. Bioneedles, which are biodegradable mini-implants, have the potential to deliver vaccines outside the cold-chain and administer them without the use of needles and syringes. In the current study, Bioneedles were filled with IPV, subsequently lyophilized, and antigenic recoveries were determined both directly after IPV-Bioneedle preparation as well as after elevated stability testing. Further, we assessed the immunogenicity of IPV-filled Bioneedles in rats and the residence time at the site of administration.

Trivalent IPV was formulated in Bioneedles with recoveries of 101 ± 10%, 113 ± 18%, and 92 ± 15%, respectively for serotype 1, 2 and 3. IPV in Bioneedles is more resistant to elevated temperatures than liquid IPV: liquid IPV retained less than half of its antigenicity after one day at 45°C and IPV in Bioneedles showed remaining recoveries of 80 ± 10%, 85 ± 4% and 63 ± 4% for the three serotypes. *In vivo* imaging revealed that IPV administered via Bioneedles as well as subcutaneously injected liquid IPV showed a retention time of three days at the site of administration. Finally, an immunogenicity study showed that IPV-filled Bioneedles are able to induce virus neutralizing antibody titers similar to those obtained by liquid intramuscular injection when administered in a booster regime. The addition of LPS-derivate PagL in IPV-filled Bioneedles did not increase immunogenicity compared to IPV-Bioneedles without adjuvant.

The current study demonstrates the preclinical proof of concept of IPV-filled Bioneedles as a syringe-free alternative delivery system. Further preclinical and clinical studies will be required to assess the feasibility whether IPV-Bioneedles show sufficient safety and efficacy, and may contribute to the efforts to eradicate and prevent polio in the future.

INTRODUCTION

Poliomyelitis is caused by any one of three serotypes of poliovirus (type 1, type 2 or type 3) that can be prevented through vaccination. Since the launch of the Global Polio Eradication Initiative (GPEI) in 1988, the global incidence of polio has reduced by more than 99% and the number of countries with endemic polio has decreased from 125 to three. While the live attenuated oral polio vaccine (OPV) is currently the vaccine of choice in developing countries, it is associated with safety concerns, i.e., reversion of the vaccine virus to a form that causes paralysis and the risk of circulating vaccine-derived polioviruses (cVDPVs) [1]. Therefore, replacement of OPV by inactivated polio vaccine (IPV) is the new strategy striving towards global polio eradication. The cost increase is a main issue in replacing OPV with the more expensive IPV. In this context, adjuvants, like the lipopolysaccharide (LPS) derivate PagL, were shown to have dose-sparing capacity for IPV prepared from attenuated Sabin strains [2].

Alternative delivery technologies, via the skin [3, 4] or mucosal sites [5, 6] might further benefit the administration of IPV in the field if they are at least non-inferior in human. These delivery technologies can have advantages, such as easy and fast administration, minimizing/eliminating the risk on needle-stick injuries or reuse of needles and/or minimal generation of waste [7, 8]. Among the alternative delivery technologies that might be used for IPV is the Bioneedle technology, which allows parenteral vaccine administration without the use of needle and syringe. Bioneedles are small hollow mini-implants from biodegradable polymers that can be filled with antigen followed by a lyophilization process. After subcutaneous delivery, the implant dissolves and thereby releases the antigen. Preclinical data with different antigens showed the feasibility of Bioneedles as vaccine delivery system [9-12]. A first phase 1 clinical study with solid Bioneedles (without antigen) revealed good tolerability [13]. Besides, if formulated properly, vaccines in Bioneedles are thermostable, which can diminish the dependence on the cold-chain [10-12].

Recently, we have developed a formulation that stabilizes IPV during lyophilization and subsequent storage at higher temperatures [14]. The aim of current study is to develop a syringe-free administered polio vaccine by using the Bioneedle technology. Therefore, Bioneedles containing lyophilized IPV were developed, thermostability was assessed by D-antigen ELISA, and the immunogenicity in rats was evaluated by determining virus neutralizing (VN) antibody titers. To investigate whether the immune response elicited by IPV-filled Bioneedles could be increased by using an adjuvant, a formulation containing the

LPS-derivate PagL was included in this study. As the kinetics at the site of administration of a dry, encapsulated vaccine may be different compared to fluid injection, we performed a real-time *in vivo* imaging study.

MATERIALS AND METHODS

Materials

The IPV used in this study is a ten times concentrated trivalent bulk at a nominal D-antigen content (expressed in D units, DU) of 400-80-320 DU/mL (for type 1, 2 and 3 respectively, Salk strains) and routinely produced by the Netherlands Vaccine Institute as described previously [15].

D-sorbitol, monosodium glutamate and magnesium chloride hexahydrate were from Sigma (St. Louis, MO). Citric acid (Sigma-Aldrich, St. Louis, MO) and Na2HPO4 (Fluka, Buchs, Switzerland) were used to prepare McIlvaine buffer. All excipients used were of reagent quality or higher grade. IRDye800CW® protein labeling kit used for the *in vivo* imaging study was obtained from LI-COR Biosciences (Lincoln, NE). The adjuvant PagL LPS is obtained through expression of the Bordetella bronchiseptica PagL gene in Neisseria meningitides LPS as described by Arenas *et al*. [16].

Methods

IPV Bioneedle production

Trivalent IPV bulk was concentrated using 10 kDa Amicon Ultra centrifugal filters (Merck Millipore, Billerica, MA) and formulated with 8% (w/v) sorbitol, 5% (w/v) monosodium glutamate and 5% (w/v) magnesium chloride in McIlvaine buffer (10 mM, pH 7.0). For the imaging experiment, IPV was labeled with fluorescent probe IRDye800 CW® according to the accompanying instructions.

Empty Bioneedles (0.9 x 12 mm, made of extruded starch) were obtained from the Bioneedle Technologies Group and filled with 5 µL of the liquid IPV formulation (with or without adjuvant) using a specially designed filling apparatus and immediately frozen on a plate at -50°C. Subsequently, Bioneedles were loaded on a Zirbus freeze-drying unit sublimator 2-3-3 with pre-cooled shelves at -50°C. The lyophilization process was based on

a process used for IPV in vials [14]. In brief, primary drying was performed at -45°C (0.045 mbar) and secondary drying by further increasing the shelf temperature to 25°C followed by a 24 h drying step at 25°C (0.01 mbar). The lyophilized Bioneedles were individually stored in vials, closed under vacuum and sealed with alu-caps.

D-antigen content of the Bioneedles directly after lyophilization and during stability testing at 45°C for four weeks and at 60°C for one week, was determined by a sandwich ELISA as described previously [17]. DU recovery was expressed relative to the liquid formulation prior to lyophilization.

In vivo fluorescence imaging

Animal experiments were conducted in accordance with the guidelines provided by the Dutch Animal Protection Act, and were approved by the Committee for Animal Experimentation of Intravacc. Female CD Hairless rats (Crl:CD-Prsshr8, Charles River, Sulzfeld, Germany) received IRDye800CW®-labeled trivalent IPV (40-8-32 DU per dose) via subcutaneous liquid injection (0.1 mL per dose) or Bioneedle administration using a sterilized trocar with mandrin as described previously (5 animals/group, unless other stated) [11]. Scans of animals, positioned in dorsal recumbence, were performed eight times during 72 h after immunization under 2% isofluran/O2 anesthesia using an IVIS Spectrum imaging system (PerkinElmer, Waltham, MA). The threshold was set using a background scan made prior to immunization. The imaging sequences were 760, 780, 800, 820 and 840 nm for emission and 710 nm as excitation. Spectral unmixing was performed to decompose the emitted light into autofluorescence and label-specific fluorescence. The data represent the quantity of the fluorophore at each pixel. Data was analyzed using the Living Imaging software 4.1 from PerkinElmer and GraphPad Prism 6.4 software. The relative fluorescence intensity (percentage of initial fluorescence) at the site of administration was calculated for each animal.

Immunization study

Outbred Wistar rats (HsdCpb:WU, Harlan Laboratories, The Netherlands) received trivalent IPV (2.7-0.6-2.1 DU/dose, 10 animals/group) with or without PagL LPS (1 µg/dose). Liquid formulations were administered intramuscularly (i.m.) or subcutaneously (s.c.) by injection in the hind limb or neck between the ears, respectively, and Bioneedles were implanted as described above. All immunizations were performed under isofluran/O2 anesthesia. Vaccinations were given on days 0 (prime) and 28 (boost), and sera collected prior to immunization on day 0, on day 21 and on day 35. On day 49, animals were sacrificed

under anesthesia by bleeding (heart puncture).

Serum polio-specific IgG and virus neutralizing (VN) antibodies against all three poliovirus serotypes were determined as described earlier [2]. Baseline VN titers reported are based on four animals receiving mock vaccine as negative control.

Statistical analysis

For comparative analysis of immunogenicity, data were tested by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer test for multiple comparisons. Probability (p) values <0.05 were considered significant. All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA).

RESULTS

IPV-Bioneedle: process and product stability

Bioneedles were filled with a trivalent IPV formulation containing excipients that are known to stabilize the antigen during lyophilization and subsequent storage. Directly after lyophilization, the D-antigen content was determined by dissolving Bioneedles, followed by a D-antigen ELISA. DU recoveries were 101 ± 10%, 113 ± 18% and 92 ± 15%, respectively for type 1, 2 and 3 (n=3).

Product stability was evaluated by incubating Bioneedles for up to one month at 45°C or one week at 60°C. Subsequently, DU recovery was determined and compared to the liquid IPV stored at the same conditions (**Figure 1**). Liquid IPV stored for 24h at 45°C showed recoveries of 10 ± 2%, 47 ± 0% and 18 ± 5% for type 1, 2 and 3, respectively, whereas the IPV Bioneedle maintained its D-antigen content at respectively 80 ± 10%, 85 ± 4% and 63 ± 4% (**Figure 1A**). One-week storage of the liquid IPV formulation at 45°C resulted in complete loss of type 1 D-antigenicity and 80% loss of type 2 and 3. After four weeks also type 2 and 3 were not detectable anymore. In contrast, Bioneedles showed about 60% type 1 recovery and complete type 2 recovery and about 50% type 3 recovery after one week 45°C. After four weeks at 45°C DU recoveries were 27±10% for type 1, 64 ± 15% for type 2, and 18 ± 13% for type 3. IPV Bioneedles containing the PagL adjuvant were also subjected to stability studies and showed DU recoveries that were comparable to those of IPV Bioneedles without PagL (data not shown).

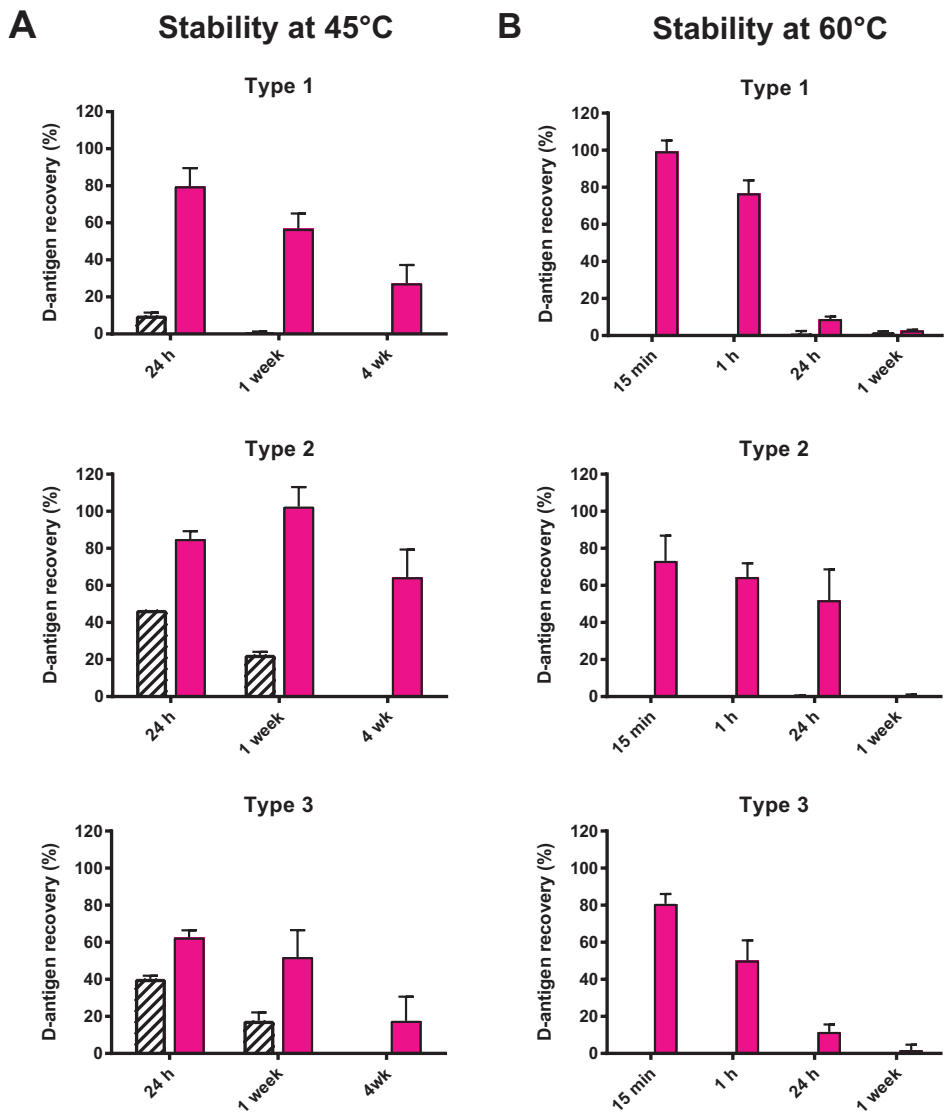

Figure 1 Thermostability of IPV Bioneedles. Liquid IPV formulations and IPV-filled Bioneedles were incubated at 45°C (A) and 60°C (B). Subsequently, for each serotype D-antigen content (D-antigen recovery) was determined by ELISA. Bars represent mean D-antigen recoveries ± SD (n=3) normalized for the D-antigen recovery directly after lyophilization.

At 60°C, the liquid formulation showed complete loss of D-antigenicity after 15 minutes, whereas no loss in DU recovery for type 1 was observed in IPV-filled Bioneedles and remaining recoveries of 73±6% for type 2 and 81 ± 5% for 3 were found (**Figure 1B**). Upon storage for a longer period at 60°C, D-antigen content decreased dramatically showing highest loss for

serotypes 1 and 3. After one week at 60°C, the Bioneedles did not contain any D-antigen anymore.

In order to test whether the formulations used in this study were able to resist real-life, unrefrigerated and thus varying conditions, liquid IPV and IPV-Bioneedles were taken on a three-week trip through Middle Eastern countries. Temperature was tracked during those weeks and DU content was determined afterwards (Figure 2B). The average temperature was determined at 26.0°C with a minimum of 17.0°C and a maximum of 46.5°C. During the trip, the IPV Bioneedles and liquid IPV were four times exposed to a temperature above 40°C (Figure 2A). For type 2 and 3, DU recoveries of 70-80% were found for both the liquid and Bioneedle formulations. However, whereas liquid IPV did not contain type 1 D-antigen, 100% DU recovery was observed in the IPV Bioneedle (Figure 2B).

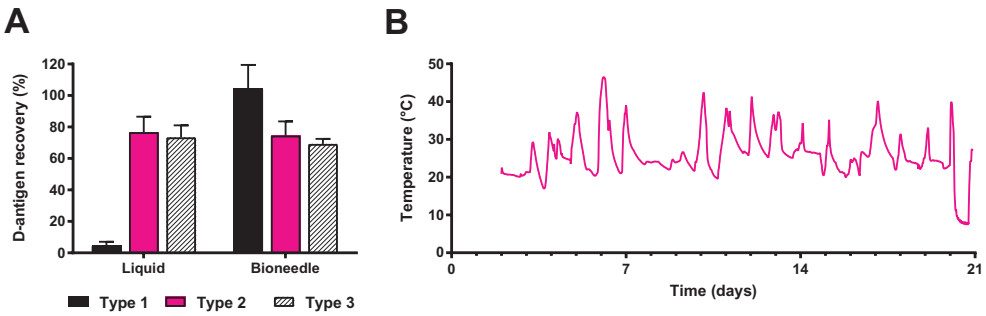

Figure 2 Stability of IPV-Bioneedles outside the cold-chain. Liquid IPV formulations and IPV-filled Bioneedles were taken on a travel through Mideast Asia countries. Temperature was logged for three weeks (A) and, subsequently, D-antigen content (D-antigen recovery) was determined by ELISA (B). Bars represent mean D-antigen recoveries ± SD (n=3) normalized for the D-antigen recovery directly after lyophilization.

Biodistribution study

To determine the kinetics of the antigen release from the Bioneedle and removal from the injection site, a real-time *in vivo* imaging study was performed over the course of time post inoculation. Within five hours after administration, the fluorescent signal from a Bioneedle filled with free IR-dye (not linked to IPV) decreased to background level (Figure 3B). Labeled IPV was removed from the injection site considerably slower than free dye irrespective of the administration method (injection or Bioneedle). Surprisingly, IPV in Bioneedles disappeared with the same kinetics as fluid injection. At 72 h post immunization, labeled IPV was not detectable at the site of administration.

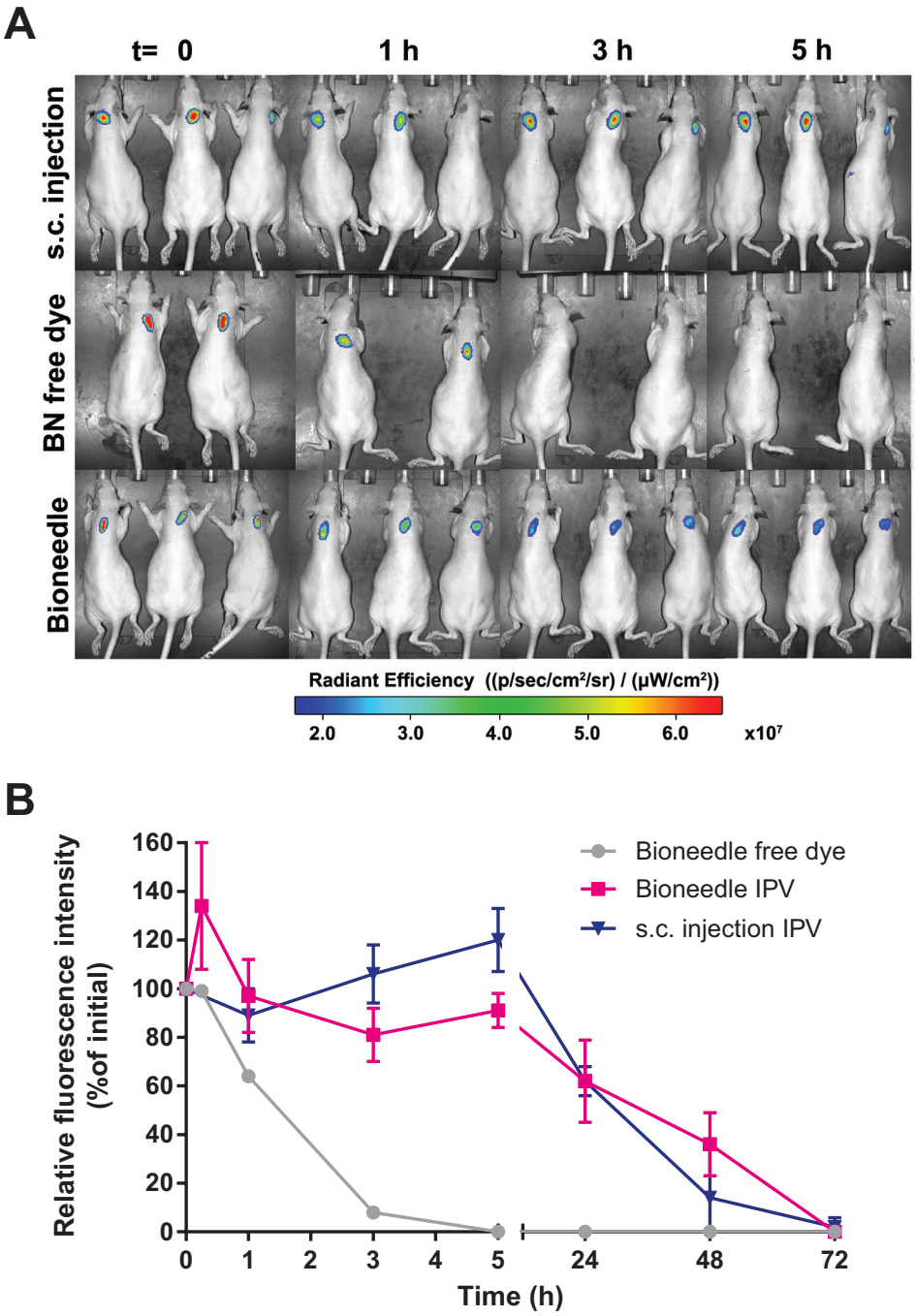

Figure 3 Kinetics of the antigen at the site of administration following IPV immunization either via liquid injection or Bioneedle insertion. Whole-body fluorescence images of nude rats in dorsal recumbence at different time points post immunization by s.c. liquid injection with labeled IPV or Bioneedle (BN) insertion with either labeled IPV or free IR dye. Depicted animals were representative for the whole group (A). The relative fluorescence intensity at the site of administration was quantified (B). Data represent means ± SD from five animals (except for BN free dye group where only two animals were used).

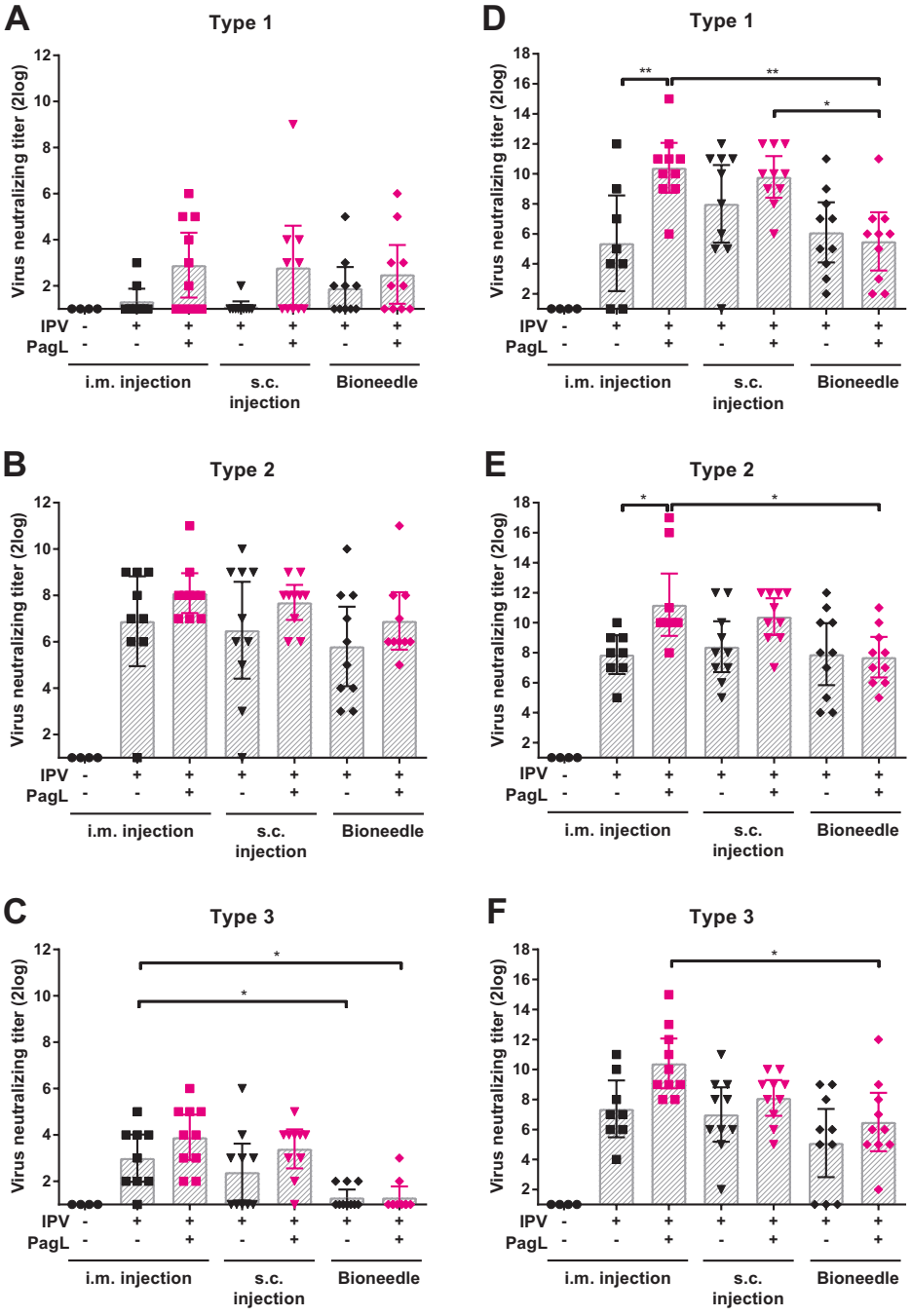

Figure 4 Mean virus neutralizing (VN) antibody titers in serum. Rats (n=10) received IPV in the absence (closed symbols) or presence of PagL LPS (open symbols) as adjuvant. Four animals received mock vaccine as negative control and are the source of the baseline VN titers. Immunizations were given via intramuscular (i.m.) or subcutaneous (s.c.) liquid injection or via subcutaneous Bioneedle insertion and the neutralizing capacity of serum antibodies was determined three weeks after prime (day 21, A-C) or three weeks after booster immunization (day 49, D-F). Asterisks indicate significant differences between groups (* $p<0.01$, ** $p<0.001$).

Immunization study

Wistar rats were immunized with IPV, delivered via either Bioneedles or liquid injection, in a prime-booster regime. Subsequently, immune responses elicited by IPV-filled Bioneedles were compared with those obtained via liquid injection.

After prime immunization, rats that received i.m. liquid injection showed low VN titers and only two of them elicited a detectable response against type 1 (Figure 4A), whereas all animals showed a VN titer against type 2 following i.m. immunization with IPV (Figure 4B). Rats receiving a prime immunization with an IPV-Bioneedle showed VN titers for type 1 and 2 that were similar to those obtained with i.m. liquid injection using syringe and needle. Although prime immunization with an IPV-containing Bioneedle induced significant lower VN titers for type 3 compared to i.m. liquid injection ($p<0.01$), it induced similar VN type 3 titers compared to s.c. liquid injection (Figure 4C). In general, higher numbers of responders were observed in the groups immunized with IPV in the presence of PagL LPS (Figure 4A-C). These responses were only significantly higher after the boost immunization for type 1 and 2.

VN titers in rats were comparable for all serotypes after booster immunization via either IPV-filled Bioneedles or i.m. IPV injection (Figure 4D-F). The route of administration had no effect on the induction of VN antibodies after booster immunization. The addition of PagL LPS showed significant improved VN titers for type 1 and 2 when administered via i.m. injection (Figure 4D-E). Serum IgG titers, which were determined by ELISA, showed similar results for all serotypes compared with the VN titers (data not shown).

DISCUSSION AND CONCLUSIONS

The current proof of concept study demonstrated the potential of an alternative delivered IPV using the Bioneedle technology. Compared with liquid IPV, IPV formulated in Bioneedles showed improved thermostability and similar kinetics at the site of injection as well as comparable immunogenicity when administered in a booster regime. Whereas improved immunogenicity was generated by addition of PagL to liquid IPV, immune responses elicited by IPV-Bioneedles were not potentiated by PagL.

The lyophilization step needed during the production of vaccine-filled Bioneedles is an important characteristic of the Bioneedle technology that might make it an interesting vaccine delivery approach without dependence on the cold-chain [10-12]. However, lyophilization of polio vaccines while maintaining their functionality showed to be challenging

[18]. Excipients are required to protect the antigen against freezing and drying stresses that occur during the lyophilization process. For production of Bioneedles containing vaccine formulations in general, the disaccharide trehalose (5% w/v) was able to fully protect tetanus toxoid [10], hepatitis B surface antigen [11] and the influenza hemagglutinin antigen (HA) during lyophilization [12]. Nevertheless, even 10% (w/v) trehalose or sucrose was not enough to maintain the D-antigen content of IPV during the freezing and subsequent drying steps [14]. The formulation used in IPV-Bioneedles was inferred from an earlier study where a combination of sorbitol, monosodium glutamate (MSG) and magnesium chloride ($MgCl_2$) was able to protect IPV during lyophilization and storage [14]. In order to obtain an appropriate viscosity for the use in Bioneedles, it was required to use the reported optimized formulation at lower amounts of the same excipients (8-5-5% instead of 10-8.5-8.5% sorbitol, MSG and $MgCl_2$, respectively) [14]. This formulation yielded similar D-antigen recoveries as compared with the optimal formulation in vials as described in the lyophilization study [14].

At temperatures above 56°C, within minutes poliovirus and vaccine are converted to C-antigen [19], which are not able to induce VN antibodies [20]. Therefore, accelerated stability was determined above and below this threshold: 45°C and 60°C. The lyophilized formulation used in this study was slightly less stable when compared to the optimal formulation in vials, both in Bioneedles and vials [14]. In contrast, whereas the lyophilized IPV formulation in vials maintained type 1 antigenicity after a one-week incubation at 45°C [14], a significant loss in type 1 D-antigen was observed for the same formulation in Bioneedles. The optimized formulation in vials had a residual moisture content above 3%, the European Pharmacopoeia limit [14]. Unfortunately, we were not able to measure the water content in Bioneedles due to interference of the Bioneedle-material with the assay. Since this formulation was less stable than the optimized lyophilized IPV formulation (in vials), we expect a higher residual moisture content for lyophilized IPV in Bioneedles, potentially resulting in the observed decrease in thermostability when compared to lyophilized IPV in vials. Nevertheless, the lyophilized IPV formulated in Bioneedles was more thermostable than the liquid IPV for all serotypes. Optimization of the IPV-Bioneedle formulation and lyophilization process, e.g., by prolongation of the secondary drying step and thereby reducing the residual moisture content, could probably further increase the stability of IPV-filled Bioneedles.

Earlier, it was suggested that Bioneedles might induce a short-term ‘depot effect’ or alter the kinetics of antigen recognition and processing, which could explain the enhanced immunogenicity of Bioneedles for some antigens [10, 12]. Some vaccine delivery systems are able to prolong the localization period of the antigen at the site of injection, thereby

slowly releasing the antigens at the injection site for a period of up to more than one week [21]. An *in vivo* imaging study was performed using infrared-dye labeled IPV to investigate whether the residence time at and release from the administration site was different between IPV formulated in Bioneedles and subcutaneously injected liquid IPV. Both administration methods (Bioneedle and liquid injection) showed a comparable release from the injection site and a similar distribution pattern over time. Within a few days, the labeled IPV in Bioneedles completely disappeared from the site of administration showing comparable clearance as subcutaneously injected ovalbumin alone in mice [22]. This demonstrated that Bioneedles did not form a depot (at least for IPV and the formulation used) at the site of injection and supports the fact that in general the immunogenicity of Bioneedle formulated antigens seemed to be comparable to injected fluid vaccine, which is an advantage in the licensing process.

Immunogenicity of IPV Bioneedles was evaluated in rats measuring VN capacity of serum, which is a surrogate marker for protection [23]. A booster regime seemed to be essential for a proper immune response similar to that induced by IPV injection in the rat model. The observed low VN titers after prime immunization were not surprising, since other IPV formulations showed also moderate immunogenicity, as indicated by low VN titers and low numbers of responders following a single vaccination (i.m.) [2, 24].

In order to increase the immunogenicity of IPV, PagL was included as adjuvant. Several studies have shown the potential of dose sparing by using adjuvants for IPV based on Salk [24-27] and Sabin strains [2, 28]. PagL LPS is able to enhance the potency of Sabin IPV after both prime and booster immunization (via the i.m. route), serotype 3 being most immunogenic [2]. Since type 3 is the most vulnerable serotype during lyophilization and subsequent storage of dried IPV [14], it was decided to include PagL in Bioneedles to compensate for possible loss of immunogenicity. The addition of PagL in the lyophilized IPV-Bioneedles did not result in improved VN titers at the dose evaluated. However, PagL was able to enhance the immune response against the poliovirus serotypes when administered via liquid injection (36-, 10-, 8-fold VN titer-increase for type 1, 2 and 3, respectively), albeit to a lesser extent as observed for Sabin IPV in a booster-regime (294-, 578- and 2352-fold for type 1, 2 and 3, respectively) [2]. The lack of comparability between wild type (Salk) IPV and Sabin IPV, which is due to their different antigenic and immunogenic properties, has been reported extensively [29-31]. The lack of adjuvant activity in the Bioneedle material may be the result of interaction between adjuvant and Bioneedle. However, another LPS-derivate, LpxL1, was able to enhance the immunogenicity of Bioneedles in combination with hepatitis B vaccine in mice [11]. Further

investigation on the PagL dose, kinetics and routing upon delivery is needed (e.g., by *in vivo* imaging). In the study reported here significant adjuvant effects of PagL included in IPV Bioneedles, at least for the evaluated dose, were not seen.

This study demonstrated the preclinical proof of concept of Bioneedles for IPV. When formulated in Bioneedles, IPV was more thermostable as compared with the liquid IPV. A clinical study in healthy volunteers showed already that solid Bioneedles without antigen were well tolerated [13]. However, several steps should be taken in the further development of this alternative delivery system for polio vaccination, including toxicity and dose finding studies. Those (pre-) clinical studies, using an approved applicator for Bioneedle administration, should prove the practical use, safety, and efficacy of Bioneedles for human vaccination, and their usefulness to strive for polio eradication and in the period thereafter.

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Conflict of interests: Gijsbert van de Wijdeven (GW) is founder of Bioneedle Technology Group (BTG) and the Bioneedle technology is proprietary to BTG. GW provided the empty Bioneedles. Intravacc did the study design, preparation of the final formulations, product characterization, immunogenicity studies and data processing. There were no restrictions on the use of generated data.