

Serum and mucosal antibody responses to inactivated polio vaccine after sublingual immunization using a thermoresponsive gel delivery system

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Abbreviations: TRG, thermoresponsive gel; dmLT, double mutant heat-labile toxin; IPV, inactivated poliovirus vaccine; ELISA, enzyme-linked immunosorbent assay; IgA, immunoglobulin A; IgG, immunoglobulin G; SL, sublingual; IM, intramuscular; CT, cholera toxin; OPV, oral poliovirus vaccine; SSI, Staten Serum Institute; TMB, tetramethylbenzidine; RT, room temperature; PBS, phosphate-buffered saline; DPBS, Dulbecco's phosphate-buffered saline; DU, D-antigen units.

Administering vaccines directly to mucosal surfaces can induce both serum and mucosal immune responses. Mucosal responses may prevent establishment of initial infection at the port of entry and subsequent dissemination to other sites. The sublingual route is attractive for mucosal vaccination, but both a safe, potent adjuvant and a novel formulation are needed to achieve an adequate immune response. We report the use of a thermoresponsive gel (TRG) combined with a double mutant of a bacterial heat-labile toxin (dmLT) for sublingual immunization with a trivalent inactivated poliovirus vaccine (IPV) in mice. This TRG delivery system, which changes from aqueous solution to viscous gel upon contact with the mucosa at body temperature, helps to retain the formulation at the site of delivery and has functional adjuvant activity from the inclusion of dmLT. IPV was administered to mice either sublingually in the TRG delivery system or intramuscularly in phosphate-buffered saline. We measured poliovirus type-specific serum neutralizing antibodies as well as polio-specific serum Ig and IgA antibodies in serum, saliva, and fecal samples using enzyme-linked immunosorbent assays. Mice receiving sublingual vaccination via the TRG delivery system produced both mucosal and serum antibodies, including IgA. Intramuscularly immunized animals produced only serum neutralizing and binding Ig but no detectable IgA. This study provides proof of concept for sublingual immunization using the TRG delivery system, comprising a thermoresponsive gel and dmLT adjuvant.

Introduction

Mucosal surfaces are portals of entry for most pathogens and consequently mucosal immune responses are an important first line of defense against pathogens causing diseases such as AIDS, tuberculosis, respiratory tract infections, diarrheal diseases, and poliomyelitis, which together cause millions of deaths annually. The mucosal adaptive immune response includes local production of secretory immunoglobulin (slgA) which is resistant to

degradation by proteases and provides protection at the pathogen's site of entry.^{1,2} Needle-free administration of vaccines directly to the mucosa—via oral, nasal, or sublingual (SL) routes—can induce mucosal responses in addition to systemic immune responses.^{3–8} Examples of marketed vaccines delivered via mucosal routes are those for polio, influenza, and rotavirus, all of which are live attenuated virus formulations.^{9–14} However, newer-generation vaccines typically are inactivated viral or bacterial preparations given by intramuscular (IM) or subcutaneous

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injection, which produce predominantly systemic immunity but poor mucosal response; thus, these routes are less effective at preventing initial infection of mucosal surfaces.^{3,7,13,15} If delivered by the oral route (and thus, swallowed), these types of vaccines have low inherent immunogenicity due to dilution in secretions, degradation by proteases, low stomach pH, and poor uptake. Lack of safe and effective mucosal adjuvants further limits the efficacy of vaccines delivered via the oral mucosal route.

The SL route of mucosal immunization addresses some of these challenges to oral use of inactivated vaccines by providing a site that is easily accessible and highly vascularized. It requires low volumes,^{2,4,7,16-18} reduces exposure to digestive enzymes, and avoids the intestinal tract where possible concurrent diarrheal illness can reduce retention time, thus preventing delivery of vaccine antigen to inductive immune sites.^{1,7,15-23} In addition, SL vaccinations have a lower risk of central nervous system complications compared with intranasal vaccines.²⁴ To enhance immunogenicity, SL vaccine formulations can include effective adjuvants such as bacterial enterotoxins; for example, cholera toxin, produced by *Vibrio cholera*, and the closely related heat-labile toxin, produced by *Escherichia coli*.^{5,25} A double mutant heat-labile toxin (dmLT) adjuvant has been developed from the latter by the insertion of 2 mutations that greatly reduce toxicity but retain strong adjuvanticity.^{26,27} dmLT is a potent mucosal adjuvant that has been used in several animal models and in a human Phase 1 clinical trial.^{7,15,18,21,26-30}

In order to provide an effective antigen dose via the SL route, residence time of the vaccine at the mucosal site must be sufficient for uptake by antigen-presenting cells while minimizing loss due to swallowing. Residence time could be increased by using a delivery system with mucoadhesive and penetration-enhancing components to retain the vaccine formulation in place. Thermoresponsive gels (TRG) are aqueous solutions at room temperature and transform into gels at typical body temperatures^{31,32}—for example, upon contact with the mucosa—and are an attractive technology for administering SL vaccines. Combining a TRG with an effective adjuvant, such as dmLT, could provide a needle-free delivery system for various inactivated and newer-generation vaccines that cannot be administered effectively via the oral route.

Polio is a highly infectious viral disease that affects mainly children. Currently, there are 3 marketed poliovirus vaccines used for childhood immunization: trivalent live attenuated oral poliovirus vaccine (OPV; the World Health Organization no longer recommends this vaccine alone), trivalent formalin-inactivated poliovirus vaccine (IPV) administered IM,³³ and Sabin IPV.^{34,35} OPV is less expensive, easy to administer, and capable of inducing both humoral and mucosal immunity; however, vaccine-associated paralytic poliomyelitis and reversion of vaccine strains to a pathogenic phenotype are major concerns that limit OPV use for poliovirus eradication.^{33,36} In addition, the World Health Organization recommends that countries currently using OPV-only vaccination add at least one dose of IPV to the vaccination schedule.³⁷ Therefore, IPV has been identified by international policymaking bodies as the only option likely to be recommended for countries wanting to continue vaccination

against polio in the post-eradication era.³⁷⁻⁴⁰ As is the case for most vaccines injected IM, the IPV does not produce significant mucosal responses, yet these responses to poliovirus have been shown to play an important role in polio disease prevention.^{41,42} Researchers are now exploring ways to induce mucosal immune responses to IPV by adding an adjuvant or changing the route of administration.^{33,36,43,44} The goal of this study was to evaluate mucosal and serum immune responses to IPV generated via SL immunization in mice using a TRG delivery system, which we define here as a thermoresponsive gel and dmLT adjuvant.

Results

TRG properties

Several TRG formulations were developed and screened based on viscosity, liquid-to-gel transition time, and retention (under the tongue) properties. In a gelation experiment, the lead formulation selected for this study traveled approximately 23% of the distance traveled by a similar phosphate-buffered saline (PBS) formulation control (Supplementary Figure S1 and Materials and methods). Rheology measurements were performed to further understand gel formulation viscosity properties (data not shown). The formulation for this study had a liquid-to-gel transition time of 5 seconds (for a 10- μ l volume) at 36° to 40°C (mouse oral cavity temperature is 36.5° to 38°C, similar to the human temperature) and retention time of 10 to 15 minutes under the tongue. The development of TRG formulations for mucosal delivery will be reported in detail elsewhere.

IPV in vivo studies

A preliminary IM dose escalation study (Supplementary Figure S2) was completed to evaluate the relative immunogenicity of a 100-fold range of trivalent IPV doses in mice. Licensed IPV preparations contain a fixed ratio of 5:1:4 for polio serotypes 1, 2, 3, respectively, due to inherent differences in immunogenicity when administered to humans. We used this ratio in our trivalent IPV studies in mice to maximize clinical relevance. No apparent dose-response relationship for neutralizing antibody titers for IPV2 or IPV3 was observed. An apparent dose response for the mean IPV-binding serum immunoglobulin (Ig) titer was observed after the first vaccine dose. Based on results from this preliminary study, a trivalent IPV dose of 1.34 D-antigen units (DU) type-1 (IPV1), 0.3 DU type-2 (IPV2), and 1.1 DU type-3 (IPV 3) (approximately 1/30 of a human dose), defined as a low dose, was selected for animal study 1. This dose was sufficient to show improvement in antibody titers compared with traditional IM vaccination due to the addition of adjuvant or to the alternate route (data not shown).

Study 1: low-dose IPV

As noted in the Methods section, the enzyme-linked immunosorbent assay (ELISA) used to determine serum immunoglobulin G (IgG) showed cross-reactivity with mouse IgA (Southern Biotechnologies mouse IgA; cat# 0106-01). Therefore, we refer to the antibodies detected by ELISA as IPV-specific serum Ig. In

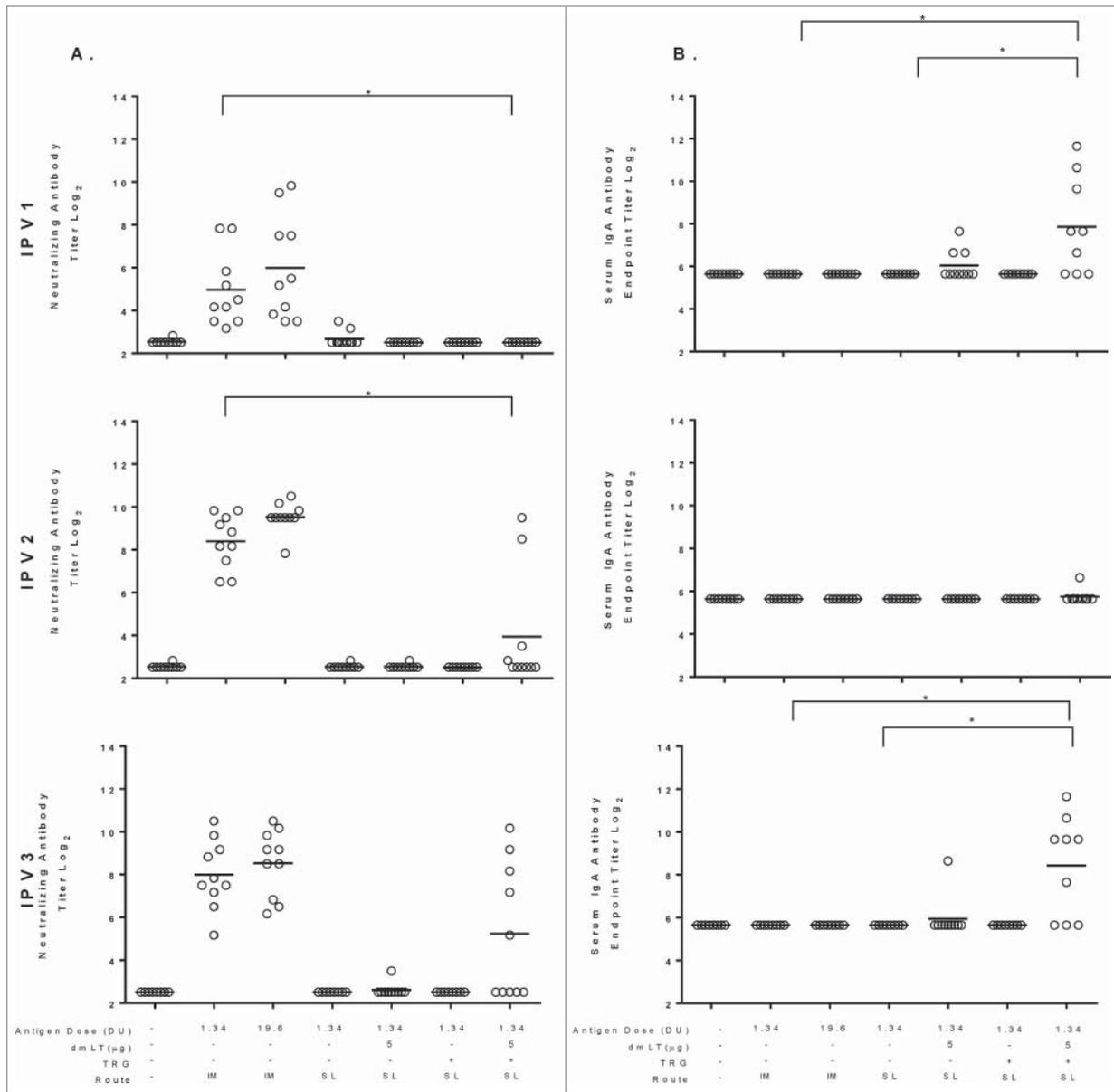


Figure 1. Study 1: Serum IPV-specific antibody responses to IPV1, IPV2, and IPV3 on day 56. Column (A) Serum neutralizing antibody response; Column (B) Serum IgA response. Responses are shown as antibody titers for each dose, formulation, and route. Antigen dose is for the IPV1 serotype. Each circle represents one animal. Horizontal lines are geometric mean titers for each group. Statistically significant differences between animals receiving IM and the TRG delivery system are shown as brackets over the 2 groups: * $p \leq 0.05$, Wilcoxon signed-rank test.

Study 1, IM-immunized animals generated the largest magnitude IPV-specific serum neutralizing (Fig. 1) and IPV-specific serum Ig (Figure S3, Supplementary material) antibody responses; however, these animals produced no measurable IPV-specific serum IgA (Fig. 1) or mucosal IgA responses as measured by salivary and fecal IPV-specific IgA (Fig. 2). Serum neutralizing antibody levels in IM-immunized animals were significantly higher than those in animals vaccinated with the TRG delivery system (Wilcoxon signed-rank test, IPV1 $p = 0.002$, IPV2 $p = 0.0039$), as was IPV2-specific Ig (Wilcoxon signed-rank test, $p = 0.002$). As defined earlier, the TRG delivery system comprises both the thermoresponsive gel and the adjuvant dmLT.

Among the 4 groups of SL-immunized animals (Table 1), the group receiving vaccine formulated in the TRG delivery system produced the strongest IPV-specific serum neutralizing (Fig. 1), serum IgA (Fig. 1), and IPV-specific serum Ig (Figure S3, Supplementary material). This group generated significantly more IPV-specific serum IgA antibodies than animals receiving SL vaccination with IPV in Dulbecco's phosphate-buffered saline (DPBS) (Wilcoxon signed-rank test, IPV1 $p = 0.0313$, IPV3 $p = 0.0313$). This group also produced the highest salivary IgA titers with the highest response to IPV3 and only a low response against IPV2 (Fig. 2). Compared with animals receiving SL immunization with IPV in DPBS, the group vaccinated with the

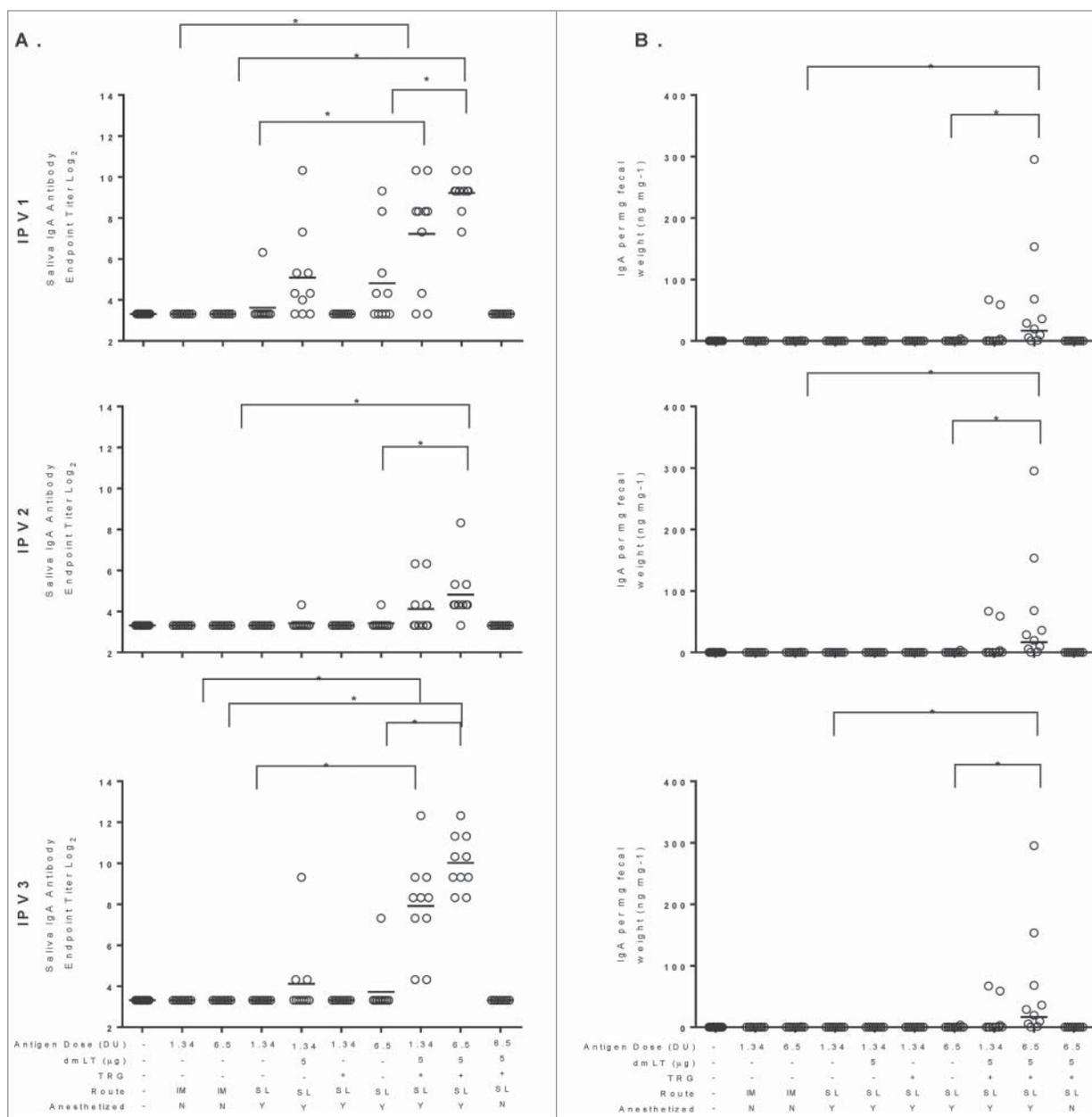


Figure 2. Study 1 and Study 2: Mucosal IPV-specific antibody responses to IPV1, IPV2, and IPV3. Column (A) Saliva IgA; Column (B) Fecal IgA. Fecal IgA data are reported as ng of IgA per mg of feces tested. Saliva and fecal samples from Study 1 (1.34 DU) were collected 14 days after the last immunization (day 56) while the samples from Study 2 (6.5 DU) were collected 7 days after the third immunization (day 49). Antigen dose is for the IPV1 serotype. Each circle represents one animal. Horizontal lines are geometric mean titers for each group. Differences in response between groups that are statistically different are shown as brackets over the 2 groups. * $p \leq 0.05$, Wilcoxon signed-rank test.

TRG delivery system produced significantly more IPV-specific salivary IgA (Wilcoxon signed-rank test, IPV1 $p = 0.0078$, IPV3 $p = 0.002$). The fecal IgA titer in the group receiving the low dose of IPV formulated with the TRG delivery system showed no difference from the other groups. SL immunization with vaccine formulated in TRG without dmLT, or formulated with only dmLT (without TRG), elicited minimal to no response for each IPV serotype (Fig. 1 and 2). Naïve animals did not generate any IPV-specific serum neutralizing, IPV-specific serum Ig, serum IgA, or mucosal IgA antibodies.

Study 2: high-dose IPV

Study 2 tested a higher dose of IPV (IPV1 6.5, IPV2 1.5, and IPV3 5.5 DU; approximately 1/6 of the human dose) and also compared SL immunization using the TRG delivery system in animals with and without anesthesia (Table 2). As in Study 1, IM-immunized animals generated the highest magnitude IPV-specific serum neutralizing (Fig. 3) and IPV-specific serum Ig (Figure S4, Supplementary material) antibody responses; however, these animals produced no detectable serum IgA (Fig. 3) or mucosal IgA response (Fig. 2). Neutralizing antibody

Table 1. IPV Study 1 dosing

Group ¹	IPV1/IPV2/IPV3 D-antigen units	Adjuvant	Formulation (Volume, mL)	Vaccination route ²	Anesthesia
1	1.34/0.3/1.1	none	DPBS (0.01)	SL	Yes
2	1.34/0.3/1.1	dmLT ³ 5 µg	DPBS (0.01)	SL	Yes
3	1.34/0.3/1.1	none	TRG (0.01)	SL	Yes
4*	1.34/0.3/1.1	dmLT 5 µg	TRG (0.01)	SL	Yes
5	1.34/0.3/1.1	none	DPBS (0.05)	IM	No
6	19.6/4.2/16.7	none	DPBS (0.06)	IM	No
7	none	none	none	none	No

¹Each group consisted of 10 animals.

²All animals were vaccinated on days 0, 21, and 42; sublingual (SL) or intramuscular (IM) routes.

³dmLT: double mutant *E. coli* heat-labile enterotoxin.

*Immunized with TRG delivery system.

levels generated by IM-immunized animals were significantly higher than those in animals vaccinated with the TRG delivery system (Wilcoxon signed rank test, IPV1 $p = 0.002$, IPV3 $p = 0.0195$), as was IPV2-specific Ig (Wilcoxon signed-rank test, $p = 0.002$).

Of the 2 groups of SL-immunized animals vaccinated under anesthesia (Table 2), the group receiving vaccine formulated in the TRG delivery system produced the greatest IPV-specific serum neutralizing (Fig. 3), serum IgA (Fig. 3), and IPV-specific serum Ig (Figure S4, Supplementary material). This group also generated significantly higher levels of IPV-specific serum IgA than groups that received SL immunization with IPV in DPBS (Wilcoxon signed-rank test, IPV1 $p = 0.0117$, IPV2 $p = 0.0156$, IPV3 $p = 0.0078$). The TRG delivery system group also displayed the strongest mucosal IgA antibody titers in salivary and fecal samples, with highest responses against IPV1 and IPV3 (Fig. 2). SL immunization with vaccine formulated in DPBS (without TRG or adjuvant) elicited minimal to no response for any IPV serotype (Figs. 2 and 3). In unanesthetized animals, vaccination using the TRG delivery system failed to elicit any immune response to IPV (Figs. 2 and 3). Naïve animals did not generate any IPV-specific serum neutralizing, IPV-specific serum Ig, serum IgA, or mucosal IgA antibodies.

Discussion

The goal of this study was to evaluate a needle-free delivery system for a licensed, inactivated trivalent poliovirus vaccine that would elicit mucosal antigen-specific IgA antibody when

delivered sublingually. The TRG delivery system produced both serum and mucosal IgA responses that were not elicited by IM immunization. In designing this system, we chose a thermoresponsive polymer containing mucoadhesive agents and added a modified bacterial toxin that functions as a safe and effective mucosal adjuvant. The gel helps protect the antigen from enzymatic degradation and minimizes loss from the oral cavity, thus maximizing uptake to provide adequate stimulation of the immune system.² The bulk component of the TRG, a Pluronic[®] compound, is a polymeric surfactant that undergoes liquid-to-gel transition at physiologic temperatures, e.g., upon application under the tongue.⁴⁵ The surfactant component provides mucosal penetration^{46,47} and is also thought to protect the antigen and adjuvant from mucosal proteases. Carbopol[®] and hydroxypropyl methylcellulose are polymers that provide mucoadhesive properties to the TRG, and Carbopol[®] also increases the permeability of mucosal cells.⁴⁸

The mucosal adjuvant dmLT was included because it enhances antigen-specific humoral and cellular immune responses in both serum and mucosal compartments after immunization via oral, SL, or intradermal routes in animal studies.^{2,18,21,26,29,30,49} One polypeptide chain of dmLT has been altered to eliminate the enterotoxicity typical for this family of bacterial binary toxins; it is considered safe and has entered human clinical trials.^{26,29} It is an advanced clinical candidate both as an antigen to protect against bacterial diarrhea and as an adjuvant. A limitation of this study in regard to evaluating dmLT is that IPV was the only antigen evaluated with the TRG delivery system. dmLT has generated higher responses as an adjuvant with other antigens, and the

Table 2. IPV Study 2 dosing

Group ¹	IPV1/ IPV2/ IPV3 D-antigen units	Adjuvant	Formulation (Volume, mL)	Vaccination route ²	Anesthesia
1	6.5/1.5/5.5	none	TRG (0.01)	SL	Yes
2*	6.5/1.5/5.5	dmLT ³ 5 µg	TRG (0.01)	SL	Yes
3*	6.5/1.5/5.5	dmLT 5 µg	TRG (0.01)	SL	No
4	6.5/1.5/5.5	none	DPBS (0.05)	IM	No
5	none	none	none	none	No

¹Each group consisted of 10 animals, with the exception of group 5, the control group, which comprised 5 animals.

²All animals were vaccinated on days 0, 21, and 42; sublingual (SL) or intramuscular (IM) routes.

³dmLT: double mutant *E. coli* heat-labile enterotoxin.

*Immunized with TRG delivery system.

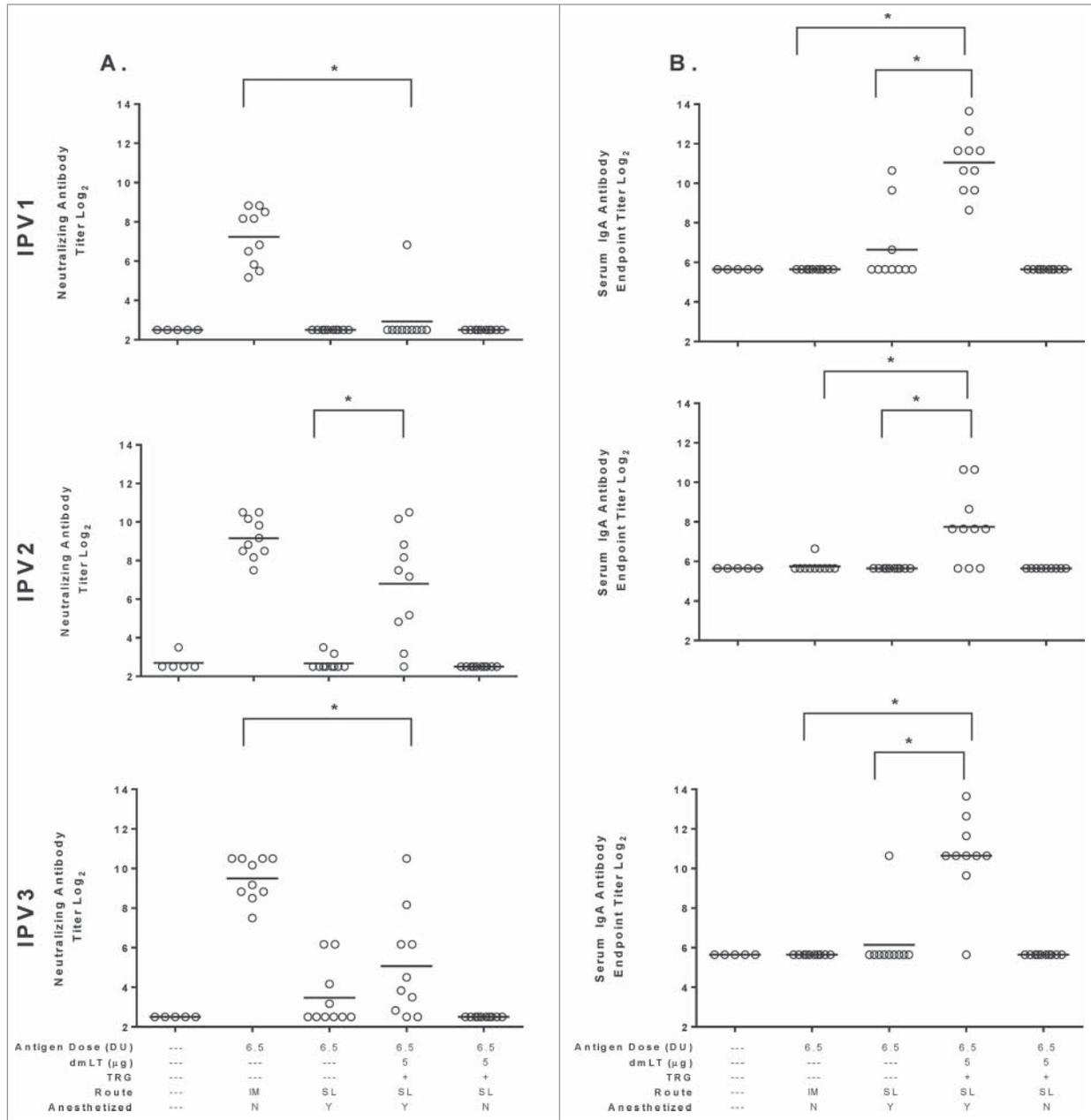


Figure 3. Study 2: Serum IPV-specific antibody response to IPV1, IPV2, IPV3 on day 49. Column (A) Serum neutralizing antibody response. Column (B) Serum IgA response. Responses are shown as antibody titers for each dose, formulation, and route. Antigen dose is for the IPV1 serotype. Each circle represents one animal. Horizontal lines are geometric mean titers for each group. Differences in response between groups that are statistically different are shown as brackets over the 2 groups. * $p \leq 0.05$, Wilcoxon signed-rank test.

lower levels of the responses generated in this study may be due to the nature of the large, complex, formaldehyde-inactivated IPV antigen.^{28,42,50,51}

Neutralizing antibody titer generally has been accepted as the correlate of protection for poliovirus vaccines and is used as the gold standard to determine effectiveness of poliovirus vaccination.^{52,53} However, mucosal IgA responses have also been reported to be important for control of poliovirus infection.^{41,54,55} Salivary and fecal IgA are considered to represent the common mucosal immune response (secretory IgA) that can

prevent initial pathogen entry, while circulating IgA and IgG form a second line of defense, mediating elimination of pathogens that have breached the mucosal surface. In addition, fecal IgA may be important in prevention of viral shedding.⁵⁴

In our studies, the combination of serum and mucosal IgA responses were observed only in animals immunized by the SL route using the TRG delivery system—not in those immunized by the IM route, nor in those immunized by the SL route with only gel or adjuvant—suggesting that both components are essential for eliciting a response when vaccinating at mucosal

surfaces.^{1,2,4,7,15,16,21} Most published studies on SL immunization in humans or animals have reported formulating vaccine antigen in PBS or medium.^{7,14} Such an approach, in the absence of gelling and mucoadhesive agents, leads to poor retention at the administration site.

Because one of our aims was to avoid the use of live attenuated polio vaccine, we used a licensed inactivated product that is intended for IM injection. Marketed trivalent poliovirus vaccines such as IPOL™ or VeroPol contain a dose level of each serotype optimized to achieve a balanced humoral antibody response and immunologic memory after IM immunization in humans in the following order: IPV 1 > IPV3 > IPV2.²² In both Study 1 and 2, the IM-immunized groups generated comparable IPV-specific serum Ig titers for each of the IPV serotypes. However, the levels of neutralizing antibody titers were similar only for serotypes 3 and 2, and lower for type 1. A possible explanation is that the IPV serotype doses and ratios selected for humans may be suboptimal in mice.²⁴ In SL-immunized animals vaccinated with the TRG delivery system, the desired balanced response for IPV-specific Ig and neutralizing antibodies to each serotype was not obtained. One potential reason for these findings is that there are differences between these 2 routes^{15,21} for antigen uptake and processing;^{11,22} hence, antigen doses may need to be adjusted for SL immunization. Another reason may be that mice respond differently than humans to these antigens.

In Study 1, not all animals immunized with the TRG delivery system generated an IPV-specific immune response, and there was a large variance in titers generated. Therefore, Study 2 was performed to evaluate a 5-fold higher antigen dose. Increasing the antigen dose increased the proportion of responding animals, and the overall antibody titers were higher, suggesting that the SL dose administered in Study 1 was suboptimal. Serum neutralizing and IPV-specific serum Ig titers for the TRG delivery system remained lower in magnitude than those in the IM control group at the higher dose in Study 2.

Study 2 also evaluated immune responses to IPV in the TRG delivery system in unanesthetized animals, in anticipation of the possibility for human application of this technology, which would not include anesthesia. None of the unanesthetized animals vaccinated with the TRG delivery system produced an immune response (serum or mucosal) for any IPV serotype. These mice vigorously tried to remove gel applied to the sublingual area by licking or swallowing, reducing the amount and time of availability of antigen at the mucosal site. While the TRG delivery system has potential to overcome barriers to effective mucosal immunization, this approach has several challenges, especially for sublingual administration in the target pediatric population, where the lack of co-operation may result in inaccurate dosing because infants and children may swallow or spit out the vaccine prior to sufficient absorption. These challenges can be partially addressed through modifications to the TRG formulation for improved absorption and retention at the site of administration. This may help in rapid uptake, giving little time for swallowing or spitting of the vaccine.

The lower systemic response from SL-immunized mice compared with IM-immunized animals in both studies could be due

to a suboptimal antigen dose for the SL route, where a portion of the dose can be lost due to dilution in saliva or involuntary swallowing even in anesthetized animals. Another explanation could be the selection of the mouse as an animal model for evaluation of SL route of immunization. Though mice are commonly used as model animals for preclinical evaluation of SL vaccines, these rodents have a keratinized SL mucosa that may reduce the uptake of antigen via this route.²⁷ While this is a possible limitation in the study design, our findings demonstrate effective induction of mucosal and serum response regardless of the possible interference of the keratinized mucosa.

Overall, this study provides proof of concept for a delivery system comprising a thermoresponsive gel and dmLT adjuvant to produce antigen-specific mucosal and serum IgA responses to a licensed IPV via SL immunization. Further use of this delivery system will require investigation of its safety/toxicity profile at the administration site, including a histological evaluation of the sublingual mucosal epithelium. Since the development of a vaccine formulation is a complex and a costly process, a systematic approach for evaluating the potential of the TRG delivery system will be needed. This would include investigation and monitoring of antigen stability with the TRG delivery components under various stress conditions of pH and temperature while maintaining the product performance (gelation and mucoadhesion). Additional studies using the TRG delivery system also will help define the best models, doses, and conditions to guide further development for eventual use in human immunization. The information gained will be valuable in forming a robust base of knowledge for using TRG delivery system as a platform technology for mucosal immunization.

Materials and Methods

TRG delivery system

Stock solutions of Carbopol® (Lubrizol; cat # CRP471PNF), hydroxypropyl methylcellulose (HPMC, Sigma; cat # 423238), and Pluronic® F127 (Sigma, cat# P2443) were prepared in purified water (Elga model PL5232). The adjuvant dmLT was supplied in lyophilized vials by Walter Reed Army Institute of Research (Lot #1735) and was reconstituted to 2 mg/mL using sterile water. Appropriate volumes of the 3 gel components were mixed with dmLT and vaccine to achieve final concentrations of 0.10% weight/volume (w/v) Carbopol®, 0.75% w/v HPMC, 15% w/v Pluronic® F127, and 0.25 mg/mL dmLT). The gelation of the lead formulation candidate at 37°C was measured as distance traveled after application to a tilted Petri dish. Briefly, a petri dish set at an angle of 75 degrees was equilibrated to 37°C for 30 minutes. Formulations mimicking the administered dose (10 µL) were applied in duplicate at room temperature, and the distance traveled was measured after 2 minutes. The control was DPBS.

Antigens

Bulk trivalent IPV was provided by Staten Serum Institute (SSI) (batch # TPA 2011-01) at concentrations of 327 D-antigen

units (DU)/mL, 70 DU/mL, and 279 DU/mL for serotypes IPV1 (Brunhilde strain), IPV2 (MEF-1 strain), and IPV3 (Saukett strain), respectively. The concentration of trivalent IPV in the final TRG delivery system was 134 DU/mL (IPV1) in Study 1 and 654 DU/mL (IPV1) in Study 2. IPV stock concentration was determined by D-antigen ELISA at SSI prior to formulating with TRG. In addition, monovalent IPV antigens were provided as assay antigens by SSI (IPV1 batch #1-2011-03; 977 DU/mL; IPV2 batch #2-2009-03; 1260 DU/mL; IPV 3 batch #3-2010-05; 1860 DU/mL).

In vivo studies

Female BALB/cj mice (6 weeks old; Jackson Laboratories Bar Harbor; cat# 000651) were used for all in vivo studies.

Preliminary study: dose selection for animal study

Seventy mice were distributed into 7 groups of 10. On days 0 and 21, animals in groups 1 to 6 were given IM doses of a ~100-fold range of trivalent IPV (19.6, 12, 4, 1.3, 0.44, or 0.15 DU, respectively, based on IPV1); the animals in group 7 were untreated. Serum samples were collected from each animal on days -1, 20, and 35 of the study. All animals were euthanized on day 35.

Study 1: Low-dose IPV

Seventy female BALB/cj mice were distributed into 7 groups of 10 (see **Table 1** for dosing information). Two groups were given IM injections with IPV formulated in DPBS (DPBS; Cellgro; cat#21-031-CV), with one group receiving a higher vaccine dose. IM vaccine did not contain adjuvant. Of the 4 SL-immunized groups, 2 received vaccine in DPBS, one with and one without dmLT adjuvant. The final 2 SL-immunized groups received vaccine in TRG, one with and one without dmLT adjuvant. All immunized groups received 3 vaccinations as detailed in **Table 1**. The seventh group of mice was a control group and received no treatments.

SL vaccination was performed by anesthetizing animals with 2.1 mg Ketamine (Mylan, generic of Ketalar, NDC#67457-108-10) and 0.132 mg Xylazine (AnaSed[®], Lloyd Laboratories NADA#139-236) mixed in 0.9% saline solution (Hospira, NDC# 0409-4888-10) intraperitoneally and pipetting vaccine under the tongue (doses and volumes shown in **Table 1**). Animals were maintained in a vertical position (holding by scruff at back of neck, head upwards, chin parallel to the floor, and mouth held open with forceps) during and for 15-20 seconds after administration. Animals were then placed prone on clean paper towels for an additional 1-2 minutes before being returned to their cage. IM injections were performed by injecting 0.05 mL (low dose) or 0.06 mL (maximum dose) into the quadriceps muscle of unanesthetized animals.

Blood was collected 20 days after the first immunization and 14 days after subsequent immunizations for analysis by ELISA (all collected via retro-orbital bleed except for the final time point, which was by cardiac puncture). Animals were euthanized 14 days after the third immunization for collection of saliva and feces for analysis of mucosal IgA responses by ELISA.

Anesthetized animals received an intraperitoneal injection of 0.05 mL of 1.8 mM pilocarpine/PBS solution (Sigma cat#P0472) to stimulate salivation. Saliva was collected with a sterile glass Pasteur pipette and transferred to a sterile tube containing 10X concentrated protease inhibitor solution (Roche Diagnostics, cat# 11697 498001). The samples were mixed and stored at $\leq -60^{\circ}\text{C}$ prior to testing.^{50,56} Large intestine content (fecal) samples were collected 14 days after the third immunization. The colon was excised from each euthanized animal and the feces were collected into a 15-mL conical tube containing 1.5 mL PBS with 0.5% Tween 20 and protease inhibitor solution. The fecal sample weight was recorded, samples were mixed vigorously for 2–5 minutes at room temperature, centrifuged at 3,000 RPM for 10 minutes at 4°C, and the supernatants (1.0 mL) were transferred to 1.5-mL tubes for storage at $\leq -60^{\circ}\text{C}$ prior to testing.

Study 2: High-dose IPV

Forty-5 female BALB/cj mice were allocated into 5 groups (see **Table 2** for dosing information). Four groups of 10 animals were immunized, while the control group of 5 animals received no treatments. One group was IM-immunized in the quadriceps muscle with IPV in DPBS. The other 3 groups received SL immunization with vaccine formulated in TRG. One of these groups received a TRG formulation without adjuvant, while the other 2 received TRG formulation with dmLT (5 $\mu\text{g}/\text{mL}$), one under anesthesia and one unanesthetized. Vaccination methods were the same as described above for Study 1. SL vaccination of unanesthetized animals was performed in a manner similar to that described for anesthetized animals except that animals were manually restrained for at least 1 minute after administration. Blood was collected 14 days after the second immunization and 7 days after the third immunization from all groups for analysis by microneutralization assay and ELISA. Euthanasia and collection of saliva and feces were performed as described for Study 1 except the collection date was 7 days after the third immunization.

Immunological Assays

Serum microneutralization antibody assay

Samples were tested using a standard microneutralization assay for antibodies to poliovirus types 1, 2, and 3 according to established protocols at the Global Polio Specialized Laboratory, Centers for Disease Control and Prevention.^{57,58} Briefly, 80–100 CCID₅₀ of each poliovirus serotype and 2-fold serial dilutions of serum were combined and pre-incubated at 35°C for 3 hours before addition of HEp-2(C) cells. After incubation for 5 days at 35°C and 5% CO₂, plates were stained with crystal violet and cell viability measured by optical density in a spectrophotometer. Each specimen was run in triplicate, with parallel specimens from one study subject tested in the same assay run, and the neutralization titers estimated by the Spearman-Kärber method⁵⁹ and reported as the reciprocal of the calculated 50% end point. Each run contained multiple replicates of a reference antiserum pool starting at a 1:32 dilution to monitor assay performance

and variation. A serum sample was considered positive if antibodies were present at $\geq 1:8$ dilution. Specimens with antibody titers $< 1:8$ were considered seronegative.

IPV Ig ELISA

For the ELISAs carried out to determine the presence of IPV-specific IgG, horseradish peroxidase-conjugated goat anti-mouse IgG (H⁺L) antibody (GE Healthcare; cat# NXA931-1ML) was used. However, initial testing showed cross-reactivity with mouse IgA (Southern Biotechnologies; cat #0106-01); therefore, we have designated the antibodies detected by this ELISA as IPV-specific serum Ig.

Serum samples were tested for Ig specific for each IPV serotype. Ig ELISA specificity for each IPV type was confirmed in a separate study (data not shown) where monovalent vaccine antigens were used to immunize animals, and no cross-reactivity was observed between IPV type-specific antibodies. Plates (Corning/Costar; cat#9018) were incubated overnight at 4°C with 0.1 mL/well of monovalent IPV antigen (2.4, 3.1, or 9.3 DU/mL of IPV1, IPV2, or IPV3, respectively) in DPBS. The plates were then washed 3 times with DPBS (Fisher; Hyclone cat# SH30378.02) /0.1% (v/v) Tween 20 (Fisher; cat# BP 337-500) using an ELx450 plate washer (BioTek) and blocked for 1 hour at room temperature (RT) with DPBS/1% (w/v) BSA (Roche; cat#100350). The plates were washed 3 times with DPBS/0.1% (v/v) Tween 20, then the serum samples and a high-titer serum standard (prepared by pooling day 63 sera from 3 groups of animals immunized by the IM route with monovalent IPV) that had been serially diluted (1:2 dilutions) in assay buffer (DPBS/0.05% Tween 20/1% BSA) were added (0.1 mL/well) and the plates were incubated overnight at 4°C. The plates were washed 5 times, then a 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (H⁺L) antibody in assay buffer was added (0.1 mL/well) and the plates were incubated for 1 hour at 37°C. The plates were washed 5 times, then tetramethylbenzidine (TMB) substrate (KPL; cat# 50-76-00) was added (0.1 mL/well) and plates were incubated at RT in the dark for 15 minutes. TMB Stop Solution was added (0.1 mL/well; KPL, cat# 50-85-06) and the OD₄₅₀ for each well was determined using a Victor3 plate reader (Perkin Elmer). End point titer results were reported as the last sample dilution having an OD₄₅₀ greater than 0.2 (which was greater than 2 times of background plus 3 standard deviations).

IPV IgA ELISA

Serum, fecal, and salivary samples were tested for IgA antibodies specific for each IPV type. Plates were coated, washed, and blocked as described above using a Skan Washer 300 plate washer (Molecular Devices). Fecal samples were diluted in DPBS to approximately 50 mg/mL prior to testing. Serially diluted (1:2) assay test samples (starting with serum 1:100, saliva 1:20, fecal fluid approximately 50 mg/mL) and controls were added at 0.1 mL/well and incubated at 4°C overnight. After washing, 0.1 mL/well of biotinylated goat anti-mouse IgA (Southern Biotechnologies; cat# 1040-08) diluted 1:5,000 in assay buffer was added and plates were incubated for 2 hours at

RT. After washing, 0.1 mL/well of avidin-peroxidase (ExtrAvidin Sigma; cat#E2886) diluted 1:1,000 in DPBS was added and plates were incubated for 1 hour at RT. Plates were developed as described for IPV-specific serum Ig and were read on a Spectramax M2 plate reader (Molecular Devices). Salivary and serum end point titers were reported as the last sample dilution having an OD₄₅₀ greater than 2.5 times the naïve control group. IgA ELISA specificity for IgA antibodies was confirmed by control wells included on each assay plate coated with mouse IgG1 at 1 µg/mL (Sigma cat #M9569). In addition, secondary antibody specificity for mouse IgA was confirmed by testing against plates coated with mouse IgA, IgG1, IgG2a, IgG2b; as expected, secondary antibodies were specific for mouse IgA at the dilution used in the ELISA. Fecal end point titers were reported for the weight of feces tested. IPV-specific fecal IgA levels were determined by comparison with a mouse IgA protein standard (Southern Biotechnologies, cat #1040-08) included as a standard on each ELISA plate. IPV-specific fecal IgA data are reported using the IgA standard to calculate the ng of fecal IgA/mg of fecal pellet weight.⁶⁰ Saliva and fecal samples are variable in their composition and represent a complex sample matrix making the quantitative antibody testing from these samples challenging.

Statistical analysis

The geometric mean titers of neutralizing, binding Ig, and binding IgA antibodies were calculated using GraphPad software (GraphPad version 6.00 for Windows, GraphPad Software, www.graphpad.com). The Wilcoxon signed-rank test was performed to compare results from animals receiving SL vaccinations with the TRG delivery system with results from IM matched-dose control animals. Separate comparisons were also made between animals receiving SL vaccinations with the TRG delivery system and animals receiving SL immunization with IPV in DPBS. P values of 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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