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Needle-free and adjuvant-free epicutaneous boosting of pertussis immunity: Preclinical proof of concept

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ABSTRACT

The limited durability of pertussis vaccine-induced protection requires novel approaches to reactivate immunity and limit pertussis resurgence in older children and adults. We propose that periodic boosters could be delivered using a novel epicutaneous delivery system (Viaskin®) to deliver optimized pertussis antigens such as genetically-detoxified pertussis toxin (rPT). To best mimic the human situation in which vaccine-induced memory cells persist, whereas antibodies wane, we developed a novel adoptive transfer murine model of pertussis immunity. This allowed demonstrating that a single application of Viaskin® delivering rPT and/or pertactin and filamentous hemagglutinin effectively reactivates vaccine-induced pertussis immunity and protects against *Bordetella pertussis* challenge. Recalling pertussis immunity without needles nor adjuvant may considerably facilitate the acceptance and application of periodic boosters.

1. Introduction

Bordetella pertussis is a respiratory pathogen readily transmitted across all ages. The widespread use of whole-cell pertussis vaccines resulted in a dramatic reduction of pertussis incidence in children [1]. Unfortunately, their reactogenicity limited their use in adolescents and adults and fears of brain damage affected public confidence, leading to their progressive replacement by less reactogenic acellular pertussis (aP) vaccines [1]. However, an increase of pertussis incidence was reported, mostly in aP vaccine-using countries [2], calling for a better control of pertussis [3]. As immunity acquired through immunization or infection is short-lived [4], particularly with aP vaccines, its reactivation requires repeat boosting [4]. Novel strategies would thus be welcome.

The use of poorly immunogenic chemically-detoxified pertussis toxin (PT) likely contributes to limit pertussis immunity [5]: it removes up to 80% of surface epitopes, reducing neutralizing antibodies, and directing B cells toward vaccine-specific rather than pathogen-specific epitopes [6]. PT with wild-type immunogenicity profile but deprived of toxicity is best achieved through genetic rather than chemical detoxification [6–8].

The skin hosts a readily accessible network of dendritic cells [9,10]. A large number of delivery devices have been developed for the transcutaneous delivery of antigens [11]. This is however limited by (i) the need to increase skin permeability through mechanical or chemical disruption of the stratum corneum and (ii) the requirement for potent adjuvants, which are not deprived of adverse reactions and safety concerns [11,12]. In contrast, a novel epicutaneous delivery system (EDS) (Viaskin®, DBV Technologies, Paris, France) currently in clinical trial for allergen desensitization (http://www.dbv-technologies.com/en/investor-relations/regulated-information/1687,First-patient-MILES) generates an occlusive chamber when applied to the intact skin: the perspiration-induced moisture releases the proteins homogenously sprayed on the membrane by electrospray, promoting their

Abbreviations: AlOH, aluminum hydroxide; aP, acellular pertussis; AUC, area under the clearance curve; *B. pertussis*, *Bordetella pertussis*; CFU, colony-forming units; DTaP-IPV, diphtheria-tetanus-acellular pertussis and poliomyelitis; EDS, epicutaneous delivery system; EPIT®, epicutaneous immunotherapy; FHA, filamentous hemagglutinin; HRP, horseradish peroxidase; LCs, Langerhans cells; OVA, ovalbumin; PRN, pertactin; PT, pertussis toxin; rPT, genetically-detoxified recombinant PT.

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diffusion across the stratum corneum toward the epidermis [13]. Previous experiments with fluorescently labeled ovalbumin (OVA) have shown that a 48 h application on intact skin is efficient for internalization and transport of the antigen, which is essentially taken up by skin Langerhans cells (LCs), to the draining lymph nodes (dLNs) and activation of local immune cells [14,15]. The role of LCs in Germinal Center formation and antibody responses was recently emphasized by Zimara et al. [16].

Here, we used genetically-detoxified recombinant PT (rPT), pertactin (PRN) and filamentous hemagglutinin (FHA) sprayed on Viaskin® to reactivate vaccine-induced pertussis immunity in the absence of adjuvant. This was addressed through the development of a murine model combining adoptive immunity transfer, immunization and *B. pertussis* challenge to assess the reactivation of memory in the absence of anti-pertussis antibodies.

2. Material and methods

2.1. Mice

Adult BALB/c female mice from Charles River (France) were kept in pathogen-free animal facilities in accordance with local guidelines. Experiments were conducted under relevant Swiss and European guidelines and approved by the Geneva Veterinary Office (GE/99/14) and the French Veterinary Services (2012_096) and Ethical Committee CEEA-26.

2.2. Antigens, adjuvants, immunizations

BALB/c mice were primed intramuscularly in both hind legs with 10 μg OVA (Grade VI, Sigma) ($n = 5$) adsorbed onto 0.25 mg aluminum hydroxide (AlOH) (gift from Novartis Vaccines and Diagnostics, Siena, Italy) (OVA/AlOH) or with 1/5th of a human dose (50 μL per hind leg) of a pediatric diphtheria-tetanus-acellular pertussis-poliomyelitis (DTaP-IPV) vaccine (Infanrix Tetra®, GlaxoSmithKline) ($n = 20$–49) containing 25 μg of PT, 25 μg of FHA, 8 μg of PRN in addition to tetanus toxoid, diphtheria toxoid and inactivated polioviruses. This protocol has been described to best correlate with pertussis vaccine efficacy in human clinical trials [17]. Mice were bled on days 27, 49 and 63 post-prime (OVA) or on days 28 and 42 (pertussis antigens) for antibody determination.

2.3. Adoptive transfer of memory B cells

Donor mice were sacrificed 6 weeks after DTaP-IPV priming and their spleens were harvested to prepare red blood cells-depleted single-cell suspensions. 90×10^6 or 50×10^6 splenocytes (in 100 μL) were transferred intravenously into each naive BALB/c recipient. Recipients were bled 6 days after transfer to quantify passively transferred PT antibodies.

2.4. Epicutaneous antigen delivery

Viaskin® EDS containing 100 μg OVA (Grade VI, Sigma) were prepared as described [14,18]. The rPT (batch number FE08/2013), FHA (batch number FE08/2013) and PRN (batch number FE08/2013) antigens produced from *B. pertussis* were provided by BioNet-Asia Co., Ltd. (Bangkok, Thailand). Antigens were purified as described [8] and resuspended in 1 mM Tris, 2 mM NaCl, 0.5 M urea, 0.5 mg/mL Brij-20, 1.4 mg/mL histidine. Antigen integrity was confirmed by SDS–PAGE.

Hair was removed from the back of mice as described [18,19]. Twenty-four hours later, Viaskin® EDS were applied for 48 h on the intact skin of their back [14,15].

Twenty eight days after priming or 1 week after adoptive transfer, mice were boosted in groups of 5 with Viaskin® EDS containing OVA (100 μg) (OVA/EDS), rPT (25 μg) (rPT/EDS), or 25 μg each of rPT, PRN and FHA (3-component pertussis (3cP)/EDS). Control mice ($n = 5$) were left unboosted or received 1/5th of a human dose (50 μL per hind leg) of an aluminum-adsorbed booster dTpa (diphtheria toxoids, tetanus and acellular pertussis) formulation (Boostrix dTpa®, GlaxoSmithKline) containing 8 μg of PT and FHA and 2.5 μg of PRN, in addition to tetanus and diphtheria toxoids. Serum samples were collected on days 7, 14 and 28 to evaluate antigen-specific antibodies.

2.5. Quantification of serum antigen-specific antibodies

Serum samples were obtained at the indicated time points (OVA: days 27, 49 and 63; pertussis: days 7, 14, 20 or 28 post-boost) for antibody detection by ELISA.

Plates were coated with 5 μg/mL OVA (Grade VI, Sigma) in phosphate-buffered saline (PBS) and blocked with 0.05% Tween and

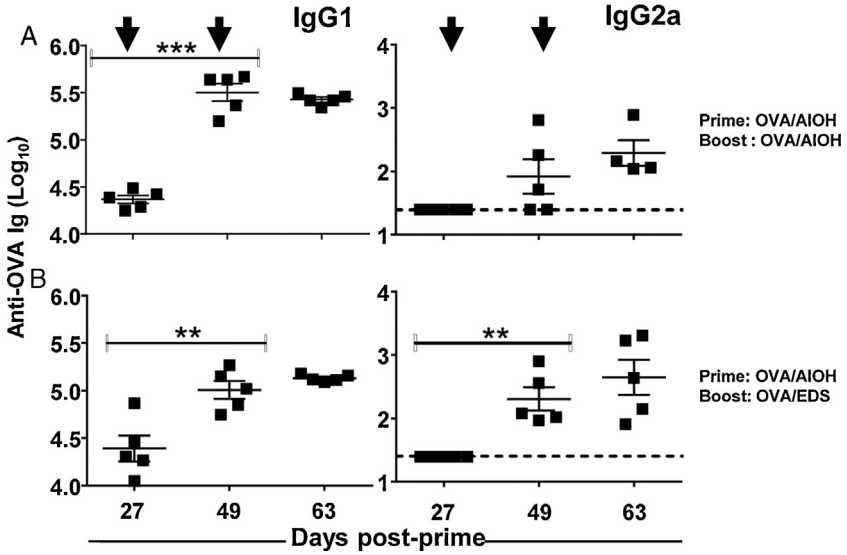

Fig. 1. Epicutaneous reactivation of OVA-specific antibodies. Naïve BALB/c mice were primed i.m. at day 0 with OVA/AlOH. Mice were boosted on days 28 and 49 (arrows) either i.m. with OVA/AlOH ($n = 5$) (A) or with OVA/EDS ($n = 5$) (B) and bled on days 27, 49 and 63 for determination of serum IgG1 and IgG2a OVA-specific antibodies compared to naïve mice ($n = 5$) (cut-off value were defined as pooled naïve mouse sera, dashed line). Data are represented as mean ± SEM, and representative of one of two experiments generating similar results, 5 mice per group. Statistical analysis was performed using the paired *t* test.

1% bovine serum albumin (BSA) (Sigma) prior to incubation with 2-fold dilutions of individual mouse serum. Plates were incubated with secondary horseradish peroxidase (HRP)-conjugated anti-mouse antibodies directed against IgG1 or IgG2a (Zymed) prior to incubation with 2,2′-azinobis (3-ethylbenzthiazolinesulfonic acid) substrate. For PT, PRN and FHA, plates were coated with 1 μg of PT or 2 μg of PRN per mL overnight at 4 °C or with 1 μg of FHA per mL for 3 h at 4 °C as described [20]. Specific antibodies were revealed with goat anti-mouse IgG (H + L)–HRP (Invitrogen, Life Technologies), IgG1 (BD Pharmingen) or IgG2a (Southern Biotech). The optical density of each well was determined with a SpectraMax (Molecular Devices) reader at 450 nm. Results were expressed by reference to serial dilutions of a titrated pool of serum (standard serum) from OVA or DTaP-IPV immunized adult mice. Antibodies below the cutoff of the assay were given an arbitrary titer of one-half of the cutoff value to allow calculation of end-point titers by reference to the standard serum included in each assay.

2.6. B. pertussis *challenge*

Streptomycin-resistant *B. pertussis* [20,21] were grown on Bordet-Gengou agar (Difco) plates supplemented with 1% glycerol (Amresco), 10% defibrinated sheep blood (Chemie Brunschwig AG) and streptomycin (100 μg/ml) at 37 °C for 24 h.

Twenty microliters of bacterial suspension (approximately 1×10^6 colony-forming units (CFU)) was instilled intranasally under anesthesia induced by Ketasol® (Graeub) and Rompun® (Bayer). Mice were sacrificed 3 h, 2 and 7 days later to quantify viable *B. pertussis* in the lungs, as described [20]. Lung homogenates were spread on agar plates and incubated for 4 days at 37 °C. *B. pertussis* colonies were counted to calculate the number of CFU per lung. The ratio of the area under the clearance curve (AUC) of immunized and naïve control mice, a measure of protective efficacy, was expressed as ΔAUC (AUC naïve mice/AUC immunized mice) [20].

2.7. *Statistical analysis*

The GraphPad PRISM (6.01) software was used for statistical analysis. Results were expressed as mean ± SEM. Statistical analysis between results obtained from various group of mice was performed using the paired *t* test or a two-tailed Wilcoxon signed rank test (*, *p* value (0.01–0.05), **, *p* value (0.001–0.01), ***, *p* value (<0.001), ****, *p* value (<0.0001)). Differences with $p > 0.05$ were considered to be insignificant.

3. Results

3.1. *Boosting antigen-specific immunity through intact skin in the absence of adjuvant*

To define whether a single Viaskin® EDS application could reactivate vaccine-induced immunity, we first used the OVA model antigen. Adult BALB/c mice were primed i.m. with OVA/AlOH, and boosted 28 days later with OVA/AlOH or with a 48 h application of OVA/EDS. Anti-OVA IgG1 and IgG2a serum antibodies increased markedly after OVA/AlOH boosting (IgG1; day 27: 4.4 ± 0.04 $\log_{10}$; day 49: 5.5 ± 0.09 $\log_{10}$, $p = 0.0007$; IgG2a; day 27: 1.4 ± 0.0 $\log_{10}$; day 49: 1.9 ± 0.27 $\log_{10}$, $p = 0.1319$) (Fig. 1A). Their increase was also significant in OVA/EDS-boosted mice (IgG1; day 27: 4.4 ± 0.14 $\log_{10}$; day 49: 5.0 ± 0.09 $\log_{10}$, $p = 0.0081$; IgG2a; day 27: 1.4 ± 0.0 $\log_{10}$; day 49: 2.3 ± 0.18 $\log_{10}$, $p = 0.0076$) (Fig. 1B). A second boost on day 49 did not further increase anti-OVA antibodies.

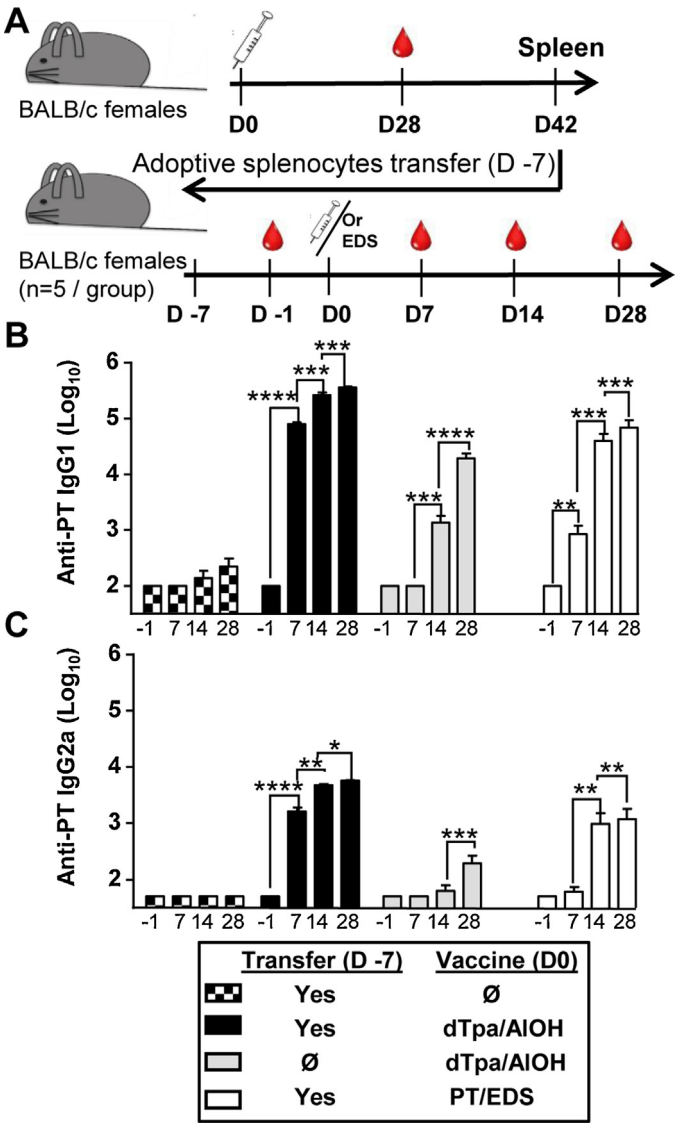

Fig. 2. Reactivation of anti-PT immune memory. (A), Cartoon illustration of adoptive transfer protocol of pertussis memory. Naïve BALB/c mice were primed i.m. with DTaP-IPV/AlOH ($n = 20$), bled 4 weeks after priming (day 28), sacrificed 42 days post-priming and their spleens harvested for adoptive transfer. 90×10^6 splenocytes per mouse were transferred intravenously into naïve BALB/c recipients (day-7). These were bled 6 days after transfer (day-1) to quantify passively transferred PT antibodies. One day later (day 0), recipients were boosted with dTpa/AlOH (i.m.) ($n = 5$) or rPT/EDS ($n = 5$), or left unboosted ($n = 5$). Mice were bled on days 7, 14 and 28 for the determination of IgG1 (B) and IgG2a (C) anti-PT specific antibodies. Control naïve mice were only primed with dTpa/AlOH on day 0 ($n = 5$). Data are represented as mean ± SEM, and representative of one of two experiments generating similar results. Statistical analysis was performed using the paired *t* test.

3.2. *Epicutaneous reactivation of pertussis vaccine-induced immunity*

To explore the capacity of Viaskin® EDS to reactivate pertussis immunity, rPT was sprayed on Viaskin® EDS and used to boost DTaP-IPV primed mice. In this experimental setting, the presence of high IgG1 antibodies elicited by DTaP-IPV/AlOH priming prevented the precise quantification of the booster response (data not shown).

To mimic the human condition in which immunization- or infection-induced memory cells persist along with low or absent anti-PT antibodies [22], we developed an adoptive transfer murine model (Fig. 2A). Anti-PT antibodies reached high titers in DTaP-IPV/AlOH-primed donor mice (6 weeks post-priming: IgG1: 5.3 ± 0.03 $\log_{10}$; IgG2a: 3.5 ± 0.05 $\log_{10}$, data not shown)

but were undetectable in recipients 6 days after splenocytes transfer (D-1), confirming the lack of antibody transfer (Fig. 2B and C). Without boosting, anti-PT antibodies increased slowly (IgG1) or remained undetectable (IgG2a). Within one week of dTpa/AlOH immunization, anti-PT antibodies increased to significantly higher titers than baseline in transfer recipients (IgG1 day 7: 4.89 ± 0.04 $\log_{10}$ vs 2.00 ± 0.0 $\log_{10}$, $p < 0.0001$; IgG2a day 7: 3.21 ± 0.07 $\log_{10}$ vs 1.7 ± 0.0 $\log_{10}$, $p < 0.0001$), but not in naïve control mice primed with dTpa/AlOH on day 0 (Fig. 2B and C). One single rPT/EDS application also reactivated anti-PT antibodies to significantly higher titers than baseline within 7 days (IgG1 day 7: 2.92 ± 0.15 $\log_{10}$ vs 2.00 ± 0.0 $\log_{10}$, $p = 0.0039$; IgG2a day 7: 1.78 ± 0.084 $\log_{10}$ vs 1.7 ± 0.0 $\log_{10}$, $p = 0.3739$), and to high titers within 2 weeks (IgG1 day 14: 4.60 ± 0.124 $\log_{10}$ vs day 28 4.84 ± 0.133 $\log_{10}$, $p = 0.0008$; IgG2a day 14: 2.99 ± 0.188 $\log_{10}$ vs day 28 3.08 ± 0.18 $\log_{10}$, $p = 0.0075$) (Fig. 2B and C). Thus, rPT/EDS triggered the rapid reactivation and differentiation of vaccine-induced PT-specific memory B cells into IgG1 and IgG2a-secreting plasmocytes.

3.3. Reactivation of protective pertussis immunity through epicutaneous immunization

B. pertussis is a complex pathogen and most vaccines include several pertussis antigens. Thus, recipient mice were next boosted with a 3-component pertussis (3cP)/EDS including rPT, FHA and PRN: this triggered within one week similarly high IgG, IgG1 and IgG2a anti-PT and PRN antibodies as a dTpa/AlOH booster (Fig. 3A and B). Anti-FHA antibodies also significantly increased within one week, although to significantly lower titers even at day 20 (IgG

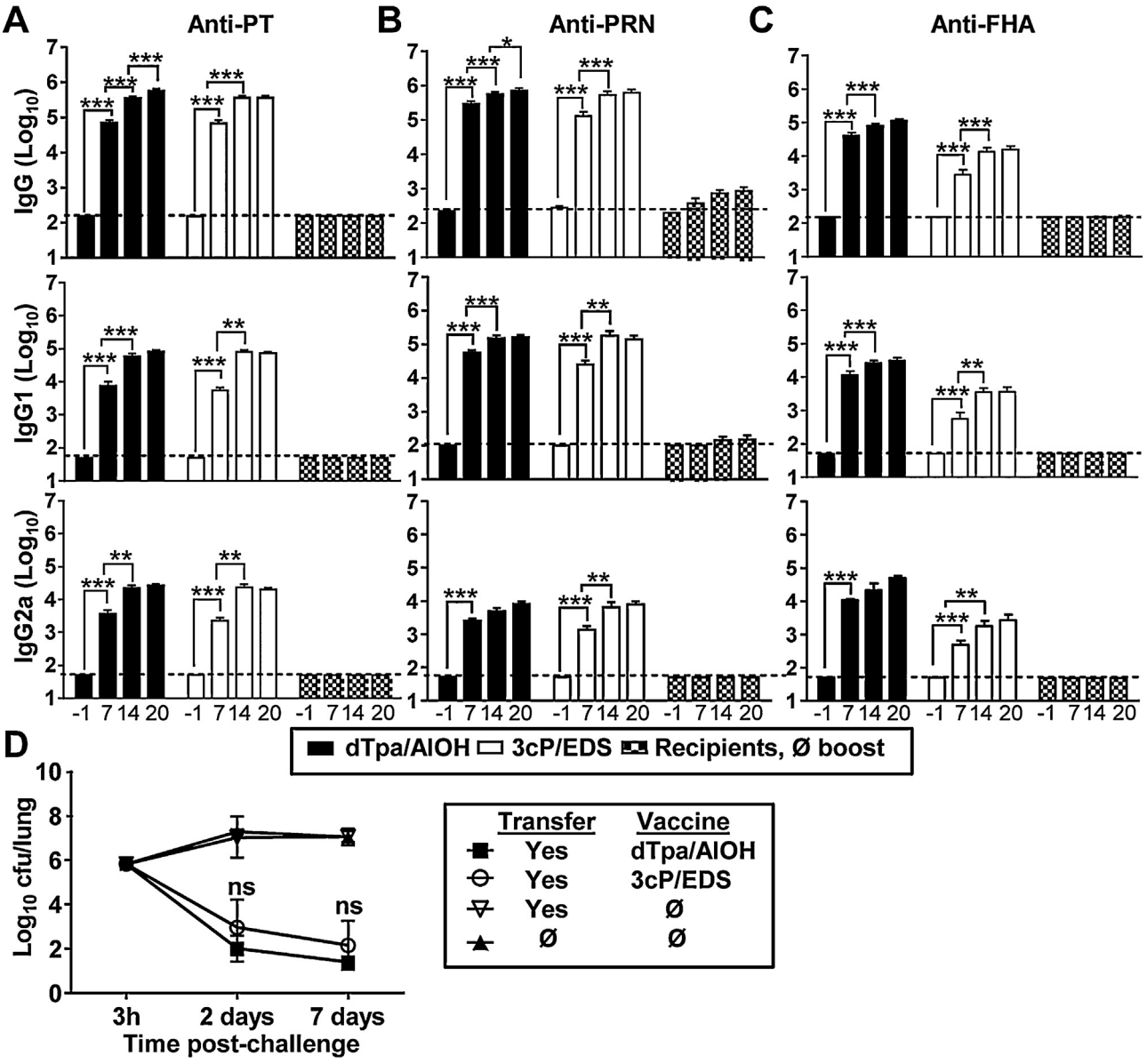

Fig. 3. Reactivation of protective pertussis immunity. Naïve BALB/c mice were primed with DTaP-IPV/AlOH and their spleens harvested 6 weeks later ($n = 49$). Recipients were transferred i.v. with 50×10^6 splenocytes per mouse and bled 6 days after transfer (day-1) to quantify passively transferred PT-, PRN-, and FHA-antibodies. One day later (day 0), recipients were boosted with dTpa/AlOH (■) ($n = 15$) or 3cP/EDS (○) ($n = 15$), or left unboosted (▽) ($n = 15$). Serum samples were collected on days 7, 14 and 20 after boosting for the determination of PT-(A), PRN-(B) and FHA-(C) antibodies. Serum from naïve mice ($n = 9$) were considered as the cut-off indicated by the dashed lines. Data are represented as mean ± SEM, and representative of one of two experiments generating similar results. Statistical analysis was performed using a two-tailed Wilcoxon signed rank test. (D), Kinetics of *B. pertussis* clearance from the lungs after respiratory challenge. Three weeks post-boost (day 21), recipient mice were challenged intranasally with *B. pertussis* and sacrificed at various intervals (5 mice per group and 3 naïve mice per time point) for evaluation of CFU counts in individual lung homogenates. Results are expressed as mean ± SD viable *B. pertussis* counts at the indicated time points.

day 20: 4.19 ± 0.11 $\log_{10}$ vs 5.06 ± 0.05 $\log_{10}$, $p = 0.0005$) than in dTpa/AlOH boosted recipients (Fig. 3C).

To confirm that 3cP/EDS reactivated functional immunity, recipient mice were challenged intranasally with *B. pertussis* 21 days after 3cP/EDS or dTpa/AlOH boosting. Bacterial loads remained high in the lungs of naive or non-boosted recipient mice (Fig. 3D). In contrast, a similarly rapid bacterial decline was observed in the lungs of recipient mice boosted with either 3cP/EDS or dTpa/AlOH prior to challenge (ΔAUC: 3cP/EDS: 21.55 ± 0.44 vs dTpa/AlOH: 16.32 ± 0.33) (Fig. 3D). Thus, protective immune memory against pertussis was reactivated through a single needle-free and adjuvant-free epicutaneous immunization.

4. Discussion

Using a new murine model, we show that pertussis immune memory may be reactivated by a single application of an adjuvant-free novel EDS and confer protective immunity.

Murine *B. pertussis* infection models present many features similar to those observed in humans [23] and *B. pertussis* clearance in immunized mice correlates with pertussis vaccine efficacy in children [24]. However, murine antibodies reach and persist at high titers after a single immunization, a condition distinct from the persistence of immune memory but waning of anti-PT antibodies prevailing in humans [22]. To reproduce this condition, we set up an adoptive transfer model in which immune splenocytes are transferred to syngeneic recipients prior to immunization and/or challenge (Fig. 2). This model (i) effectively transfers immune memory, demonstrated by the rapid (1 week) and strong increase of IgG antibodies following boosting; (ii) avoids the persistence of serum antibodies and (iii) does not transfer a significant number of plasma cells, as reflected by the minimal increase of antibodies in the absence of boosting. Antibody responses and protection induced by the immunization of recipient mice thus reflect the reactivation of transferred memory B cells and their differentiation into IgG-producing antibody-secreting-cells. Further work will decipher how various types and numbers of memory B and T cells influence boosting capacity.

Using this adoptive transfer model, we demonstrate that epicutaneous immunization may reactivate vaccine-induced immunity on intact skin and in the absence of adjuvant. Our adjuvant-free approach was based on the distinct activation requirements of memory and naive B cells [25–27]: memory B cells expressing Ag-specific surface IgG bind antigens with a higher affinity [28] and thus require lower antigen concentrations and lower T cell help than naive B cells [29]. We postulated that this may enable the reactivation of immune memory in the absence of adjuvant, a hypothesis confirmed with OVA and 3 pertussis antigens.

The efficient uptake, internalization and transport of antigens to the draining lymph nodes by skin LCs [14,15] is likely to play a critical role [16]. Skin stripping prior to EDS application classically increases antigen diffusion [14,15]. However, it did not enhance anti-OVA responses (data not shown), suggesting that antigen delivery is not a limiting factor of the Viaskin® EDS. This is also supported by the similar boosting capacity of single or repeat EDS applications. The combination of 3 antigens did not interfere and rather increased anti-PT antibodies, perhaps as a result of enhanced bystander T cell help from follicular T helper cells. Further investigations will define the exact mechanisms at play, the minimal amount of antigen required on the EDS and the optimal ratio of rPT, FHA and PRN.

The effective delivery of antigens through the Viaskin® EDS and unique properties of rPT are likely to both contribute to the success of our approach. Indeed, the use of rPT improves protective efficacy against wild-type strains [6]. A recent clinical study indicated that boosting with rPT was safe and induced significantly higher neutralizing antibodies than current acellular pertussis vaccines (BioNet forthcoming data, http://www.businesswire.com/news/home/20141015006848/en/BioNet-Update-Recombinant-Acellular-Pertussis-Vaccine-BioJapan#.VGK69skVji0).

Our study has limitations. Murine models do not recapitulate all the features of human pertussis infection, which is best studied in nonhuman primates or human challenge studies [30]. Human skin is thicker and less hairy [31] than murine skin: despite a comparable density of dendritic cells, their deeper localization in humans may limit their targeting [32]. However, the Viaskin® EDS was demonstrated as a sensitive tool for the diagnosis of cow's milk [13] and house dust mites [33] allergy in children, for which it is in clinical use in France. Encouraging results have been collected for its use in epicutaneous immunotherapy (EPIT) [15,18,19], and the safety and efficacy of Viaskin®-mediated EPIT against peanut allergy was demonstrated in a recent phase IIb clinical trial [34].

Our observations therefore raise considerable hopes that this needle-free, adjuvant-free EDS may prove an efficient antigen-delivery-system for periodic boosting of pertussis immunity, addressing an unmet need. It could increase injection safety, be rapidly administered by non-injection-trained staff, improve compliance by reducing fears and injection-associated pain and address the unfounded but growing concerns related to the repeat use of aluminum adjuvants, especially during pregnancy. A proof-of-concept Phase I clinical study is thus in preparation.

Contributors

Conceived and designed the experiments: B.M.G., L.M., P.H.L., P.H.B., C.A.S. Performed the experiments: B.M.G., L.M., V.D., C.S.E., F.A. Analyzed the data: B.M.G., L.M., P.H.L., C.A.S. Contributed reagents/materials/analysis tools: L.M., P.H.B., H.T.P., J.P. Wrote the paper: B.M.G., L.M., C.S.E., F.A., H.T.P., J.P., P.H.L., P.H.B., C.A.S.

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Conflict of interest

H.T.P. is the Chief Executive Officer of BioNet-Asia, Co., Ltd., Thailand. BioNet-Asia, Thailand has filed a patent for its recombinant *B. pertussis* and has developed a recombinant aP vaccine. J.P. is the Chief Scientific Officer at BioNet-Asia, Co., Ltd., Thailand, and one of the inventors of BioNet-Asia patent on recombinant *B. pertussis* under filing. J.P. is a board member at Biotech Tools, Belgium. V.D. and L.M. are employed by DBV. P.H.B. is a founder and Chief Executive Officer at DBV and received Honoraria from DBV. DBV has recorded a patent for its Viaskin®. C.A.S., P.H.L. and L.M. are the inventor of a DBV/UNIGE patent on epicutaneous boosting. P.H.L. received Honoraria as a scientific adviser for DBV. The authors have no other conflict to declare.

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