Needle-free Epicutaneous For t 2 DNA Vaccine is Effective for Preventing and Treating Biting Midge (*Forcipomyia taiwana*) allergy in a murine model

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Abstract

Backgrounds: Allergen-specific immunotherapy (ASIT) is capable of inducing immune tolerance to the corresponding allergen, thereby enabling treatment of the root cause of the allergic disease. As the treatment course of protein-based vaccines for ASIT is time-consuming, an easily administered epicutaneous anti-allergic DNA-based vaccine is an attractive method, especially during the COVID-19 pandemic. Methods: We established a mouse model allergic to the biting midge, Forcipomyia taiwana, to test the concept of the epicutaneous DNA vaccine. The experiments were designed using two approaches: therapeutic and prophylactic. Mice were patched with 25 μg For t 2 DNA vaccine patches for one hour with a total of three treatments spaced one week apart. Scratch bouts after For t 2 allergen challenge were used as a clinical surrogate of itch and biomarkers for allergic inflammation were examined by ELISA, RT-PCR, and histopathology. Results: We found that after epicutaneous DNA vaccination, the mice significantly improved with respect to allergen-induced scratch. For t 2-specific IgE, mRNA, and protein of IL-13 and eosinophils infiltration in the targeted skin decreased. For t 2-specific -IgG2a and mRNA expression of FOXP3 increased. Conclusions: We demonstrated the first needle-free epicutaneous DNA vaccine patch that is effective in preventing as well as treating biting midge allergy in a murine model. The mice showed improvements in their allergic condition both clinically as well as in allergic inflammation. The mode of this anti-allergic DNA vaccine may have the potential for use in other specific immunotherapies for other allergens.

1—INTRODUCTION

Allergen-specific immunotherapy (ASIT) remains the only treatment that is capable of inducing immune tolerance to the corresponding allergen and potentially treating the root cause of the allergic disease.[1-3] Currently available vaccines for ASIT, either by subcutaneous injection or sublingual administration, are all protein-based. As the treatment course of protein-based vaccines for ASIT is time-consuming, an easily administered epicutaneous anti-allergic DNA-based vaccine is not only an attractive alternative, but also important in patient care, especially during the COVID-19 pandemic.[4,5]

The skin has been known for centuries to be a unique site for immunization. Delivering a vaccine via the skin has been shown to elicit similar or even higher immune responses compared to intramuscular injection.[6,7] Several epicutaneous protein-based placebo-controlled clinical studies have been conducted to investigate the effectiveness and safety of treatment for grass pollen rhino-conjunctivitis, cow's milk allergy, and peanut allergy. Though the exact protocol of this new delivery method varies among studies, the results of pilot studies of this convenient delivery system are generally positive.[8-10]

The biting midge, *Forcipomyia taiwana* , is the most prevalent cause of biting insect allergy in Taiwan. It is a tiny hematophagous midge that attacks en masse. As many as 60% of exposed individuals develop intense

itchy reactions to the bites.[11,12] The midge is widely distributed throughout Taiwan and southern China. The hematophagous midges of the same genus have also been reported worldwide, from tropical to temperate places.[13-17]Among the 11 identified allergens, For t 2 is the most predominant allergen for *F. taiwana,* with 75% of midge-allergic patients showing specific IgE to For t 2.[18,19]

A previous study by the authors showed that a DNA vaccine encoding the For t 2 midge allergen was able to prevent the development of biting midge allergy in a mouse model.[20] We also demonstrated in recent studies that a DNA vaccine is able to treat mice with an established midge allergy.[21]

Though only two injections are required for the For t 2 DNA vaccine developed in our previous studies, a vaccine that could be delivered via the skin would be more practical and would make self-administration at home a possibility. A convenient delivery system also potentially reduces the total cost of treatment as patients do not need to go to the hospital for this specific therapy.

In this study, we demonstrate the first needle-free epicutaneous DNA vaccine patch that is effective in preventing as well as treating biting midge allergy in a murine model.

2—Materials and methods

2.1—Expression and purification of the recombinant For t 2 protein from *E. coli* for sensitization of mice

E.coli -expressed For t 2 recombinant protein (rFor t 2) was purified by rapid affinity column chromatography as previously described.[22] After using a His-tag affinity column, rFor t 2 was further purified using Endotoxin Detoxi-Gel (Pierce, Rockford, IL, USA) and sterilized with a 0.22 μm syringe filter (Millipore, Billerica, MA, USA). The endotoxin content was determined using an E-TOXATE kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Finally, the protein concentration was determined by the Coomassie brilliant G-250 protein-dye binding method of Bradford with bovine serum albumin as a standard according to manufacturer's procedures (Bio-Rad, Hercules, CA, USA).

2.2—Preparation of pVAX-For t 2 plasmid for vaccination

For t 2-encoding fragment (GenBank accession EU678971) was amplified by PCR and the PCR products were subcloned into pVAX1 (Life Technologies, Carlsbad, CA) . pVAX1 is a 3.0 kb plasmid vector designed to conform with the Food and Drug Administration (FDA) document published in 1996 (Points to consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications. PTC document Docket No 96-N-0400). The eukaryotic expression plasmid pVAX-For t 2 was then propagated in*E. coli* DH5α and large-scale purification was performed with the EasyPrep EndoFree Maxi Plasmid Extraction kit according to the manufacturer's instructions (Tools, New Taipei City, Taiwan).

2.3—Detection of For t 2 protein expression in the skin

Intradermal expression of the For t 2 proteins was evaluated. The For t 2 DNA vaccine was skin-patched for 1 hour then removed from the abdominal skin of the mouse. Twenty-four and 48 hours after being vaccinated, two mice were sacrificed, respectively. The skins from the vaccinated sites were collected and immersed in 10% formalin overnight at room temperature. Briefly, 5-μm sections were subjected to detection with rabbit anti-For t 2 (1200-fold dilution) and then incubated with peroxidase-conjugated goat anti-rabbit IgG and stained in a DAB solution using Bond automatic system (Leica, Newcastle, UK).

2.4—Experimental design of therapeutic and prophylactic DNA vaccination by epicutaneous application

Female 6-week-old BALB/c mice were purchased from the National Laboratory Animal Center, Taiwan and raised under specific pathogen-free conditions. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Taichung Veterans General Hospital (approval no. La-1081655). The mouse model of *F. taiwana*allergy obtained by sensitization to the major allergen, For t 2, was established as previously described.[20,22]

The pilot dose-finding schedule is summarized in Supplementary Figure S1. Groups of mice (n=6) were first sensitized with 2 intraperitoneal injections (IP) of 10 μg rFor t 2 absorbed by 2 mg alum adjuvant, with a 1-week interval between each injection. Topical delivery of DNA antigens was performed as follows. To elicit a specific immune response, on day 13, the hair of the abdominal area of the mice was removed using a depilatory (Supplementary Figure S2). In addition, tape stripping was used to disrupt the skin barrier at days 14, 21, and 28 before epicutaneous DNA vaccination. The three groups of skin-vaccinated mice (SV10, SV25 and SV50) received the For t 2 DNA vaccine three times using a volume of 10-50 μl administered to the pre-shaved skin at doses of 10, 25, and 50 μg for 1 hour, respectively. The non-vaccinated (sham) group served as the negative control and received a topical application of the same volume of PBS alone. All groups of mice were challenged intradermally (ID) with 3 doses of rFor t 2 for 3 consecutive days between days 59-61. Blood samples were collected bi-weekly from the submandibular vein. The scratching behaviors of the mice were video recorded on days 0 and 61, and the mice were sacrificed on day 63.

Next, we investigated the effect of 25 μg For t 2 DNA/patch on the treatment and prevention of biting midge allergy. Timelines of the therapeutic and prophylactic vaccination and the grouping of experimental mice are summarized in Figure 1A, 1B and 1C, respectively. For the therapeutic vaccination, three treatments using skin patches containing the DNA vaccine were given after sensitization to For t 2 (Figure 1A). For the prophylactic approach, mice received three DNA patches prior to sensitization with recombinant For t 2 (Figure 1B).

2.5—Measurement of total IgE and specific For t 2 antibodies by ELISA

The total IgE levels were measured using IgE mouse ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Ninety-six-well plates (Nunc) were coated overnight at 4 with 5 μg/mL monoclonal antibody. All of the following steps were performed at room temperature. After washing twice with PBS/0.05% Tween 20, the plates were blocked with PBS plus 1% BSA for 2 hours and then incubated for 2 hours with prediluted serum (1:10) or 2-fold serial dilutions of mouse IgE standards. After washing, detection antibody diluted 1:250 was added and incubated for 2 hours. The plates were washed, and then streptavidin-horseradish peroxidase conjugate diluted 1:400 was added. After incubation for 30 minutes, tetramethylbenzidine (TMB) was added, and the plate was incubated for 10 minutes. The reaction was stopped with 1 M phosphoric acid, and absorbance was measured with an ELISA reader (TECAN, Austria) at 450 nm.

The specific IgG1 and IgG2a to For t 2 were determined by in-house ELISA with the required antibodies purchased from BD Pharmingen (San Jose, CA, USA). Microtiter plates were coated with rFor t 2 for 2 hours at 37. After washing with PBST, plates were blocked with 2% BSA for 2 hours at room temperature. Sera were diluted (1:100 for IgG2a or 1:1000 for IgG1) in PBST and incubated at room temperature for 2 hours. The plates were washed and incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG1 or IgG2a for 2 hours and developed by adding ABTS solution (Sigma). The optical density was then analyzed on an ELISA reader at 415 nm.

2.6—Evaluation of scratching behavior of immediate-type reaction

At the beginning and the terminal days of every experiment, the scratching behaviors were videotaped for 1 hour starting immediately after the intradermal challenge with rFor t 2. Counts of scratching were made using video playback. The observation of scratching behavior was performed as described previously.[20,21]

2.7—Histological examination of delayed-type reaction

On day 61, experimental animals were intradermally challenged with rFor t 2 and videotaped immediately. After 48 hours, the mice were sacrificed and the abdominal skins from the challenge sites were removed and placed in 10% formalin overnight at room temperature. Briefly, tissues were embedded in paraffin, cut into 5-μm sections, de-paraffinized, dehydrated, and stained with hematoxylin and eosin (H&E). All slides were recorded under a Hamamastu nanozommer 2.0 HT slide scanner (Hamamastu, Japan) at 400-fold magnification. The numbers of different infiltrated cells in dermis were counted at 400-fold magnification by

light microscopy.

2.8—Measurement of cytokine production

Splenocytes were cultured in 24-well plates at a concentration of 1×10^6/mL and stimulated with various doses of rFor t 2 at 37 for 3-5 days. The culture supernatants were collected at each time interval and stored at -20 until use in the cytokine assay. The levels of IL-10, IL-13, and interferon-γ (IFN-γ) in the culture supernatants of splenocytes were measured with murine ELISA kits (eBioscience, San Diego, CA, USA) according to the instructions of the manufacturer.

2.9—RNA isolation and real-time quantitative PCR

Total RNA was extracted from splenocytes of individual mice after stimulation with 1 μg/mL of rFor t 2 or medium alone for 3 days using TRIzol reagents. First-strand cDNA synthesis was carried out using SuperScript III kit (Invitrogen, Carlsbad, CA). The analyzed target genes were IL-10, IL-13, IFN-γ, and FOXP3, and these specific primers are given in supplementary Table 1. PCR reactions were run on an ABI StepOnePlus machine (Life Technologies) as follows: an initial 10-minute step at 95 followed by 40 cycles of 95 for 15 seconds and 60 for 1 minute. The fluorescent signal from SYBR Green was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. Data were imported into an Excel database and analyzed using the comparative cycle threshold method with normalization of the raw data to β-actin.

3—Results

3.1—*In vivo* translation of For t 2 proteins in skin and spleen after epicutaneous vaccination

To detect the For t 2 protein translation *in vivo* , mice were sacrificed after removal of the vaccine patches for 24 and 48 hours, respectively. The vaccinated skin and spleen were removed for IHC with specific For t 2 polyclonal antibody. As shown in Figure S3, For t 2 proteins were detected in the targeted skin and the immune organ spleen at 24 hours and became more significant at 48 hours**.**

3.2—Epicutaneous DNA vaccine ameliorated For t 2-induced scratch behaviors in mice

To evaluate the therapeutic effect of For t 2 DNA patch vaccine, all mice were sensitized intra-peritoneally (IP) with *E. coli* -expressed recombinant For t 2/alum according to a previously published model. Then, on days 14, 21, and 28, mice received the For t 2 DNA patch onto the pre-shaved skin at doses of 10, 25, and 50 μg (SV10, SV25 and SV50) for 1 hour. After the final challenge, the scratching behaviors (surrogate marker of an itch) were immediately videotaped for 1 hour. In the non-vaccinated group, the mean number of scratching bouts was >300 (Figure S4). All For t 2 DNA patch-treated groups showed significantly fewer scratching bouts compared with the non-vaccinated group.

3.3—Patch delivery down-regulated Th2 antibody but up-regulated Th1 antibody

ELISA was used to determine the effects of the DNA patch vaccine on the total IgE and For t 2-specific IgG2a levels in the sera. Figure S5A revealed that the total IgE of SV25 group was significantly decreased. However, the For t 2-specific IgG2a of SV25 group was significantly elevated (Figure S5B).

3.4—Patch delivery suppressed the infiltration of eosinophils in the skin lesion

Based on our previous study, delayed reactions of midge allergy involve multiple inflammatory cell infiltration, especially eosinophils.[12,19] Histological examination was conducted using skin tissues derived from mice given different doses of For t 2 DNA patches (Figure S6A). The data clearly showed that doses of 10 and 25 μg/patch markedly decreased the infiltration of eosinophils on rFor t 2-challenged abdominal skins, as shown in Figure S6B.

Taken together, our pilot study revealed that the optimal dose of For t 2 DNA vaccine was 25 μg/patch. We therefore performed the rest of the experiments with this dose.

3.5—25-µg DNA patches ameliorated For t 2-induced scratch behaviors with both therapeutic as well as prophylactic approaches

For midge-allergic patients, the primary goal of treatment is to provide relief from the annoying itchy allergic reaction to the midge bite. The For t 2 DNA patches at 25-µg significantly ameliorated midge allergen-challenge-induced scratch bouts either in mice already sensitized to the midge (therapeutic) or before being sensitized (prophylactic) compared with the non-vaccinated groups, resulting in some instances to numbers of scratch bouts as low as those observed in the non-sensitized groups (NS), as shown in Fig 2A and 2B, respectively.

3.6—25-µg DNA vaccine patches suppressed total IgE production and increased For t 2-specific IgG2a with both therapeutic and prophylactic approaches

The left panels of Figure 3A and 3B show a scatter plot for the final total IgE profiling from an individual mouse at week 6 or 8, respectively. For both the therapeutic and the prophylactic approach, SV25-T and SV25-P groups showed significantly less total IgE production compared with the NV-T and NV-P groups. Moreover, the SV25-T group had higher level of For t 2-specific IgG2a antibodies than the respective non-vaccinated groups at week 6. The levels of For t 2-specific IgG2a at SV25-P group reached statistical significance at week 8 (right panels of Figure 3A and 3B).

3.7—25-µg DNA vaccine patches significantly decreased the infiltration of inflammatory cells in skin tissues with both therapeutic and prophylactic approaches

Figure 4 depicts the pathologic changes in dermis in response to midge allergen For t 2 challenge. In the NV-T and NV-P mice of Figure 4A and 4B, For t 2 allergen challenge significantly increased total and eosinophil cell count compared to the NS mice. In contrast, total and eosinophil cells infiltrations were suppressed in the SV25-T and SV25-P groups.

3.8—25-µg DNA vaccine patches decreased the expression of Th2 cytokine IL-13 from the For t 2-stimulated splenocytes with both therapeutic and prophylactic approaches

Spleens were harvested from all groups of mice and stimulated with rFor t 2 allergens to determine the cellular immune responses after the epicutaneous DNA vaccine. Significant down-regulations of IL-13 mRNA expression and protein secretion were detected in both SV25-T (Figure 5A) and SV25-P (Figure 5B) groups in comparison with the NV-T and NV-P groups. Interestingly, the Th1 cytokine IFN-γ exhibited an increasing trend in the SV25-T group and decreased in the SV25-P group, though no statistically significant differences in comparison with the NV-T and NV-P groups were detected (right panels of Figure 5A and 5B).

3.9—The therapeutic approach upregulated FOXP3 mRNA expression but not the prophylactic approach

As shown in left panels of Figure 6A and 6B, there was no significant difference in the expression of IL-10 mRNA and protein expression of the splenocytes after rFor t 2 allergen stimulation between NV-T and SV25-T as well as NV-P and SV25-P groups. However, the transcriptional factor of regulatory T cells forkhead box protein P3 (FOXP3) as shown in the right panel of Figure 6A was significantly increased in SV25-T group in comparison with the NV-T group. This change was not observed between the NV-P and SV25-P groups.

4—Discussion

This is the first study to demonstrate an epicutaneous anti-allergic DNA vaccine that is effective in treating an established allergic condition and preventing the development of an allergic condition using biting midge allergy as a model. After epicutaneous DNA vaccination, not only the For t 2 allergen-induced itchy response decreased significantly, the biomarkers for allergic inflammation, including allergen-specific IgE, allergen-specific IgG2a, allergen-challenge induced eosinophil infiltration in the skin, Th2 cytokines from the splenocytes, and regulatory T cell-related transcription factors also suggested that immune tolerance was induced after three patches of the epicutaneous DNA vaccine.

It is known that only lipophilic molecules with a molecular weight below 500 Dalton are able to pass though the stratum corneum of the skin.[23] Therefore, vaccination into the dermal component usually requires penetration techniques, such as microneedles, jet injection, DNA tattooing, thermal ablation, or addition of chemical enhancers to modify the stratum corneum.[6,24-26] Our data showed that though the molecular weight of the For t 2 DNA vaccine is as high as 4000 base pairs, it is able to penetrate the dermal barrier and translates the corresponding protein in the targeted skin as well as the spleen of the vaccinated mice. It is possible that the DNA vaccine passes the epidermis via the hair follicles as the skin is tape-stripped before epicutaneous vaccination.[27]Further experiments may be required to explore the delivery mechanism of this epicutaneous DNA vaccine.

This epicutaneous anti-allergic DNA vaccine induced a similar immune response to that of the intra-muscular anti-allergic DNA vaccine investigated in our previous studies.[20,21] As we did not compare the effect of intra-muscular and epicutaneous DNA vaccine directly in this study, it is not known whether the epicutaneous DNA vaccine is superior to the intra-muscular modality with regard to its effectiveness. However, the convenience of epicutaneous delivery is certainly a major advantage.

This study did not address the duration of effectiveness of the epicutaneous DNA vaccine and whether it is comparable to the intra-muscular modality or the conventional protein-based vaccine. Further studies on the duration of the protective effect of epicutaneous DNA vaccine are required.

Recent studies surveying the conditions of allergen-specific immunotherapy among allergic clinics during the COVID-19 pandemic showed that a significant number of patients discontinued their ASIT[28] and up to 60% of allergists stopped receiving new ASIT patients.[4] This situation highlights the need for an easily administered anti-allergic vaccine with a short course, such as the epicutaneous DNA vaccine presented in the current study.

In conclusion, we demonstrate the first needle-free epicutaneous DNA vaccine patch that is effective in preventing as well as treating biting midge allergy in a murine model. The mice showed improvements in their allergic condition both clinically as well as in terms of allergic inflammation. The mode of this anti-allergic DNA vaccine may have the potential for application in specific immunotherapies for other allergens.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest with regards to this study.

Author contributions

LMF and CYH designed studies, interpreted the results and wrote the article. WCS performed experiments and analyzed data. LSJ gave technical support and revised the manuscript.

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Figure legends

Figure 1 Schedule of therapeutic (A), prophylactic (B), vaccination and grouping (C) for For t 2 DNA with skin patches. Time points of sensitization with rFor t 2/alum, videotaping, and sacrificing are indicated.

Figure 2 Scratching bouts of mice from (A) therapeutic and (B) prophylactic groups. The scratching counts were recorded for 20 minutes after induction by intradermal injection of rFor t 2 at the endpoints of the two experiments. $^{**}p < 0.01$ by one-way analysis of variance with the Bonferroni multiple range test.

Figure 3 Change in serum total IgE and For t 2-specific IgG2a antibodies of mice from (A) therapeutic or (B) prophylactic groups as indicated in weeks. * denotes $p < 0.05$, **denotes$p < 0.01$, ns denotes not statistically significant by one-way analysis of variance with the Bonferroni multiple range test.

Figure 4 Effects of For t 2 vaccine on histopathology of challenged skins from (A) therapeutic or (B) prophylactic groups by H&E staining. Figures showed the representative abdominal skin sections obtained 48 hours after intradermal challenge under 100 x and 400 x light microscope. Moreover, the infiltrating inflammatory cells were quantified under 400-fold view from each group. The statistical significance of differences between groups was assessed by the Bonferroni multiple range test. ** denoted $p<0.01$, ns denotes not statistically significant.

Figure 5 mRNA and protein expression levels of Th2 and Th1 cytokines in For t 2-stimulated splenocytes from (A) therapeutic and (B) prophylactic groups by real-time PCR and ELISA. Real-time PCR data are expressed as mean of increasing folds ± SEM. The statistical significance of differences between groups was assessed by the Bonferroni multiple range test. * denotes $p<0.05$, ** denotes $p<0.01$, ns denotes not statistically significant.

Figure 6 mRNA and protein expression levels of IL-10 and mRNA of the FOXP3 transcription factor in For t 2-stimulated splenocytes from (A) therapeutic or (B) prophylactic groups by real-time PCR and ELISA, respectively. Real-time PCR data are expressed as mean of increasing folds ± SEM. The statistical significance of differences between groups was assessed by the Bonferroni multiple range test. * denotes $p<0.05$, ns denotes not statistically significant.

Figure S1 Dose finding schedule of For t 2 DNA patch vaccine on rFor t 2-sensitized mice in the pilot study.

Figure S2 The representative skin images show before (A) and after (B) patch application. The For t 2 DNA vaccine was delivered onto the pre-shaved abdominal skin through a Fin chamber for 1 hour.

Figure S3 H&E and immunohistochemotry (IHC) staining with rabbit anti-For t 2 polyclonal antibody of skin and spleen sections under 400x light microscope. For t 2 protein expression of skin and spleen after vaccination was examined after skin patch for 24 and 48 hours in mouse abdominal skin.

Figure S4 Counts of scratching bouts from each group of mice before (day 0) and after (day 61) induction by intradermal injection of rFor t 2. The scratching behavior was videotaped and data represent the number of scratches for 20 minutes after allergen challenge. $^{**}p < 0.01$; $^{***}p < 001$ by one-way analysis of variance with the Bonferroni multiple range test.

Figure S5 (A) Total IgE and (B) For t 2-specific IgG2a antibodies in the sera at week 9 determined by ELISA. Results are mean ±SD of 5 mice from each group. * denoted $p < 0.05$, **denoted$p < 0.01$, ns denotes not statistically significant by one-way analysis of variance with the Bonferroni multiple range test.

Figure S6 A. Effects of For t 2 vaccine on histopathology of abdominal skins by H&E staining. Figures showed the representative skin sections obtained 48 hours after intradermal challenge under 100 x and 400 x light microscope. B. The infiltrating inflammatory cells were quantified under 400-fold view from each mouse (n=5 per group). The statistical significance of differences between non-vaccinated group and vaccinated groups was assessed by the Bonferroni multiple range test. * denotes $p<0.05$, ** denotes $p<0.01$, ns denotes not statistically significant.