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High immunogenicity of nicotine vaccines obtained by intradermal delivery with safe adjuvants

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Abstract

Immunotherapy for tobacco addiction may offer a safe, alternative treatment if the immunogenicity of the current nicotine vaccines can be improved. We show here that intradermal (ID) immunization induces the production of antibody directed against nicotine (NicAb) at a much higher level than conventional intramuscular (IM) immunization. The magnitude and duration of NicAb production was further increased robustly by non-inflammatory laser vaccine adjuvant (LVA), slightly inflammatory monophosphoryl lipid A (MPL) or a combination of MPL and CpG adjuvants. Consequently, significantly fewer vaccination doses were required to attain a high level of NicAb production for an extended period of time and reduce nicotine entry into the brain in the presence of LVA, MPL or MPL/CpG adjuvant, respectively. Yet, the potency of these adjuvants to augment ID nicotine vaccine immunogenicity came at the expense of local skin reactivity, with LVA causing little skin reaction and MPL/CpG stimulating overt skin irritation. These observations underscore a necessity of a balance between optimal adjuvant potency and undesired local reactivity. In summary, our study presents a novel approach to significantly improve nicotine vaccine immunogenicity by a combination of safe cutaneous vaccine adjuvants with ID immunization.

Keywords

nicotine vaccine; adjuvant; intradermal delivery; immunogenicity; addiction; immunotherapy

Introduction

According to the center for disease control and prevention, cigarette smoking causes more than 443,000 deaths and 8.6 million severe illnesses each year in the USA, leading to an estimated annual economic loss of \$197 billion. Nicotine is the major component of cigarette and other tobacco products that initiates and maintains nicotine addiction. Effective

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treatment of nicotine addiction is urgently needed for both economy and public health. Besides conventional counseling, nicotine replacement therapy, and other medication therapy with low efficacy and high relapse rate [1, 2], immunotherapy with nicotine vaccines may offer a safe, alternative treatment for tobacco addiction by induction of anti-nicotine antibody (NicAb) to bind nicotine in the circulation and block or slow its entry into the brain [2-5].

Nicotine is a small molecule and must be conjugated to a large carrier to efficiently elicit a specific immune response. The current nicotine conjugate vaccines advanced to clinical trials include NicVAX, NicQb, and TA-Nic with nicotine conjugated to recombinant *P. aeruginosa* Exoprotein A (rEPA), virus-like particles formed by the coat protein of the bacteriophage Qb, and recombinant cholera toxin B, respectively [3, 6]. Even in the presence of FDA-approved, the most widely used aluminum salt-based adjuvant (alum), NicQb did not increase the abstinence rate significantly over placebo in a phase II clinical trial in 2009 mainly due to variable anti-nicotine antibody production and NicVAX failed in two phase III clinical trials in 2011 for the same reason [7]. The low vaccine efficacy was also reflected in early phase clinical trials of NicQb and NicVAX, in which only 30% of vaccinees developed a relatively high NicAb and it was this subgroup who showed a statistically increased abstinence rate after 5-7 monthly immunizations as compared to placebo [4, 5]. Other vaccinees with low or medium NicAb titers had a similar abstinence rate as placebo [4, 5]. A poor immunogenicity of the nicotine vaccine and a high variability of the NicAb titer even in the presence of Alum adjuvant may be the primary reason behind these failures. Nevertheless, a positive correlation between the NicAb level and an abstinence rate highlights the necessity of improving nicotine vaccine immunogenicity to increase a clinical response rate.

One way to increase nicotine vaccine immunogenicity may be to deliver the vaccine via the skin rather than the muscular tissue. Skin is an attractive target for vaccine delivery due to the presence of large amounts of resident antigen presenting cells (APCs). Clinical immunization of different vaccines (e.g., influenza vaccine, anthrax vaccine, and hepatitis B vaccine) consistently shows the advantage of the ID over IM route in induction of immune responses [8-12]. Apart from the inoculation route, adjuvants can greatly enhance vaccine-induced immune responses. Unfortunately, alum is not suitable for cutaneous vaccination due to its skin irritation effect, in spite of its long safety record for IM immunization [13]. Another adjuvant monophosphoryl lipid A (MPL) was recently approved in human papillomavirus vaccine in USA [14-16]. There are also other adjuvants currently under clinical trials, like unmethylated CpG oligonucleotide (CpG), water-in-oil emulsion Montanide ISA 720, and Imiquimod (R837) [17-19]. However, most of these adjuvants have been only tested in IM immunizations. Their reactogenicity in the skin and potency to enhance ID nicotine vaccine immunogenicity remain largely unknown. In addition, we have shown that brief illumination of the injection site prior to vaccination can significantly enhance immunity provoked by protein-based vaccines without incurring any additional side effect [13, 20]. In the current study, we evaluate how ID immunization in the presence of various adjuvants, either alone or in different combinations, affects the immunogenicity of a nicotine vaccine and skin integrity.

Materials and Methods

Animals

Male BALB/c mice (6-8 weeks) were purchased from Charles River Laboratories (Wilmington, MA) and housed in conventional cages in the animal facility of Massachusetts General Hospital. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (80mg/kg) and xylazine (20mg/kg) during hair removal, laser treatment, and

immunization as previously reported [20]. All procedures were in compliance with institutional, hospital, and NIH guidelines.

Adjuvants

Laser vaccine adjuvant (LVA) was elicited as our previous report [20]. MPL adjuvant was purchased from Sigma (L6895, St. Louis, MO). TLR7/8 agonist R837 and TLR9 agonist CpG 1668 was ordered from Invivogen (San Diego, CA). Alum (Imject®) was obtained from Pierce (Rockford, IL). Water-in-oil Montanide ISA 720 adjuvant was kindly provided by SEPPIC, Inc. (Fairfield, NJ).

Local reactogenicity

BALB/c mice were treated with LVA or ID injected with the following adjuvants in 20µl volume: 25µg MPL, 30µg CpG, 100µg R837, 50% Alum (v/v), 70% Montanide ISA 720 (v/v), or combinatorial MPL/CpG, MPL/R837, MPL/Alum adjuvants at the same amount of individual adjuvant as above. Adjuvant formulations were prepared freshly just before use per the manufacturer's instruction. Five days after adjuvant injection, photos of adjuvant-injection site were taken and skin tissues were dissected and subjected to standard histological analysis.

Immunization

Conjugation of 6-carboxymethylureido nicotine to keyhole limpet hemocyanin (Nic-KLH) was previously described (Pravetoni et al., 2012). For IM immunization, the posterior thigh muscle was injected with 2.8µg Nic-KLH alone or in combination with Alum adjuvant in a final volume of 20µl as prepared as above. For ID immunization, the low dorsal skin was ID injected with 2.8µg Nic-KLH with or without LVA, MPL, or MPL/CpG. MPL (25µg) or MPL/CpG (25µg/30µg) was mixed with Nic-KLH just before injection. LVA was only used in the primary immunization. Immunizations were repeated every two weeks for 7 times for IM group, 4 times for Alum+IM group, 3 times for ID or LVA+ID group, and 2 times for MPL+ID and MPL/CpG+ID group to explore the relative potency of fewer ID immunizations in the presence of various adjuvants to more IM immunizations in the presence or absence of Alum adjuvant.

Serum NicAb titer

A small blood sample was collected by tail vein bleeding and serum was prepared and stored at -80°C until analysis. NicAb titer was measured by enzyme-linked immunosorbent assay (ELISA) using 1 µg/ml of 6-carboxymethylureido nicotine conjugated to bovine serum albumin (Nic-BSA) as a coating antigen. NicAb titer was calculated as serum dilution factor when OD_{490nm} was 0.2 over controls as previously reported [20].

Nicotine challenge and nicotine assays

After the final immunization, mice were continuously monitored for 3 more months for long-term NicAb production. The immunized and control mice were weighed and challenged by intravenous injection of 0.01 mg/kg nicotine on a weight basis diluted in PBS within one week after the final blood sample collection. Five minutes after challenge, mice were decapitated and trunk blood was collected. Serum was separated and serum volume was further measured. The brain was also collected and weighed. The tissue nicotine levels were quantified by gas chromatography and expressed as nicotine quantity per gram of brain or per milliliter of serum [6].

Statistics

Student's *t*-test or Mann-Whitney test was used to analyze the difference between two groups. One-way analysis of variance (ANOVA) was used to analyze the difference among multiple groups. P value was calculated by PRISM software (GraphPad, San Diego, CA) and considered significant when it was less than 0.05.

Results

ID immunization is superior to IM

We first evaluated whether ID nicotine vaccination without adjuvant could elicit more potent immune responses than IM. As shown in figure 1, ID immunization induced a significantly higher NicAb titer than IM injection over a broad nicotine vaccine dosage from 2.8 to 16.8 μ g. NicAb titer was 15-, 7-, and 5-times higher in the ID group than in the IM group when 2.8, 11.2, and 16.8 μ g Nic-KLH was administered, respectively.

Local reactogenicity caused by various adjuvants

We next determined which adjuvant was safer to further boost ID nicotine vaccination. As shown in figure 2, R837, MPL/CpG and MPL/R837, and MPL/Alum caused the most severe skin reactions with a lesion size of 4-6 mm in diameter, concurrent with skin ulceration (upper panel) and infiltration of a large number of inflammatory cells (lower panel). Alum and to a lesser extent Montanide ISA 720 provoked a similar lesion size of 4-6 mm in diameter (upper panel) and alum but not Montanide ISA 720 inflamed the injected site significantly (lower panel). The presence of apparently void areas in Montanide ISA 720-injected skin (arrows, lower panel) might be caused by the deposition of the water-in-oil emulsion adjuvant. Different from these adjuvants, MPL or CpG alone gave rise to mild local skin reactions with a lesion size of only about 2 mm in diameter (upper panel) with more inflammatory cells infiltrated into MPL-injected skin than CpG-injected skin (lower panel). Among the adjuvants tested, LVA stood up as the safest cutaneous adjuvant, causing little skin irritation or inflammation (figure 2), in agreement with the ability of LVA to enhance DC motility not inflammation [13, 20]. Importantly, a combination of LVA with MPL or CpG did not exacerbate the local skin reaction as compared to MPL or CpG alone (figure 2), paving the way for further increasing the efficacy of ID immunization by combining LVA with MPL or CpG.

Synergistic effects between LVA and MPL or MPL and CpG

MPL has been approved in human papillomavirus vaccine [16] and it is well known to induce DC maturation, distinct from LVA that enhances DC motility. This, in line with the safety of LVA/MPL for cutaneous immunization (figure 2), prompted us to explore any additive or synergistic effect between LVA and MPL in vaccine adjuvanticity. As can be seen in figure 3A, when 40 μ g OVA or the same amount of OVA admixed with MPL was ID injected into sham or laser-illuminated skin, OVA-specific IgG titer was increased by approximately 4, 10, and 21-fold with LVA, MPL and LVA/MPL adjuvant, respectively. A 2-fold and 5-fold increase in OVA-specific antibody production in LVA/MPL group than in MPL or LVA group suggests a synergistic effect between LVA and MPL adjuvant.

The synergistic effect was next demonstrated with nicotine vaccine as shown in figure 3B in which NicAb titer was increased from 120 ± 10 in the vaccine alone group to 503 ± 170 in LVA group, 1596 ± 130 in MPL group, or 3949 ± 352 in LVA/MPL group, corresponding to an approximate 4-, 13-, and 33-fold increase by LVA, MPL, and LVA/MPL, respectively. A more vigorous vaccine adjuvanticity or a 76-fold increase was attained when MPL was combined with CpG and mixed with nicotine vaccine, in agreement with synergistic effects

of these two adjuvants previously reported [21, 22]. This combination, however, caused more severe local skin reactogenicity (figure 2).

Immune-enhancing and long-term effect of cutaneous adjuvants

We further addressed the potency of LVA, MPL, LVA/MPL, and MPL/CpG in enhancement of ID nicotine vaccine immunization. As shown in figure 4A, seven IM vaccinations induced a NicAb titer at ~10k, whereas it took only three ID immunizations to yield a higher NicAb titer at ~14k. The three-ID immunization was comparable to four-IM immunization with alum adjuvant in terms of the magnitude and longevity of NicAb titer. Incorporation of LVA into the primary ID immunization further increased NicAb titer to ~35k (figure 4A). Remarkably, a more robust augmentation was seen when MPL or MPL/CpG was employed, which increased NicAb titer to ~70K or 140K, respectively, after just 2 immunizations (figure 4A). This enhancement represents a 5-fold or 10-fold increase in NicAb titer than that obtained with 3 ID immunizations or a 7-fold or 14-fold increase than that induced by 7 IM immunizations (Table 1). NicAb titer generally peaked 2 weeks after the final immunization and fluctuated 15% above or below the peak level in the next 2-4 weeks (figure 4A). The peak NicAb production dwindled gradually but significantly ($p<0.05$) after ~3 months with ID immunization without adjuvant or IM immunization with or without alum adjuvant (figure 4A). In contrast, the peak NicAb level was well sustained for more than 4.5 months (~19 weeks) or beyond in the presence of LVA, MPL or MPL/CpG adjuvant in ID immunization (figure 4A and Table 1).

Inhibition of nicotine entry into the brain

To determine the efficacy of nicotine vaccine-induced antibody in a blockade of nicotine entry into the brain, the immunized and control mice were intravenously challenged with 0.01 mg/kg nicotine, equivalent on a weight basis to the nicotine absorbed from two-thirds of a cigarette by a smoker [23]. No significant difference in body weight, brain mass, or serum volume was found among non-immunized and immunized groups as analyzed by one way ANOVA, suggesting that all immunization regimens did not introduce any systemic adverse effects in the animals (data not shown). As shown in figure 4B, brain nicotine concentrations reached 24.0 ng/g at 5 minutes after the challenge in non-immunized mice, but significantly diminished to 15.2 or 14.4 ng/g in mice after 7 IM or 3 ID immunizations ($p<0.05$ and 0.01, respectively), corresponding to a 37% or 40% inhibition on brain nicotine entry. Incorporation of Alum adjuvant in IM immunization significantly reduced brain nicotine level to 10.1 ng/g ($p<0.01$), corresponding to a 58% inhibition on brain nicotine entry. Incorporation of LVA only in the primary immunization of the 3-ID immunization regimen reduced brain nicotine entry by 60% ($p<0.001$), and the presence of MPL or MPL/CpG inhibited brain nicotine entry by 69% or 77%, respectively, as compared to non-immunized control mice ($p<0.01$ and 0.001, respectively). As compared to ID group, incorporation of LVA, MPL, and MPL/CpG further significantly reduced nicotine entry into the brain by 34%, 48%, and 62%, respectively ($p<0.001$ for each group).

The reduction in the brain nicotine level was concomitant with a proportional increase in a serum nicotine concentration. As shown in figure 4C, serum nicotine levels were below the detection level (<4 ng/ml) in all the non-immunized mice and 3 out of 4 IM immunized mice, which was arbitrarily assigned to 1 ng/ml for simplified comparison. Serum nicotine level was 4.3 ng/ml in the ID group, increasing by 167% to 11.5 ng/ml in the LVA+ID group ($p<0.05$), by 130% to 9.9 ng/ml in the MPL+ID group ($p<0.05$), or by 284% to 16.5 ng/ml in the MPL/CpG+ID group ($p<0.01$) as shown in figure 4C. Alum adjuvant increased the serum nicotine level to 11.3 ng/ml from 1.9 ng/ml in the IM group (figure 4C).

The correlation between serum NicAb titer and brain or serum nicotine levels or between brain and serum nicotine levels was analyzed by linear regression analysis. A strong correlation was found between brain and serum nicotine levels (figure 5A, $r^2=0.46$, $p<0.001$), between serum NicAb titer and brain nicotine level (figure 5B, $r^2=0.54$, $p<0.001$). There was also a weak correlation between the NicAb titer and serum nicotine level (figure 5C, $r^2=0.28$, $p<0.01$).

Discussion

We show in the present study that nicotine vaccine immunogenicity can be greatly improved by combination of ID immunization with adjuvants such as LVA, MPL, or MPL/CpG. Similar to other vaccines [8-12], the nicotine vaccine induced a much higher NicAb titer when administered by ID than IM route (figure 1 and 4A). The level of NicAb production was further increased by addition of vaccine adjuvants. Among the three adjuvants analyzed, MPL/CpG was the most potent, but also caused the most severe local reactions. This is not surprising in that the skin is considered highly sensitive to immune responses due to its richness in APCs. LVA is not as potent as MPL or MPL/CpG, but it is the safest adjuvant, causing little skin irritation. LVA also does not involve injection of any chemicals or substrates. Hence, the use of LVA in ID nicotine vaccination would raise little concern about long-term side effects even after repeated use. This may be important because nicotine addiction requires repeated or prolonged immunotherapy.

To the best of our knowledge, this is the first report of efforts to improve nicotine vaccine immunogenicity by combination of ID injection and vaccine adjuvants. The novel approach not only augmented the magnitude of NicAb production but also prolonged the peaking NicAb level for an extended period of time (figure 4), which may be helpful to achieve continuous abstinence. In addition, ID delivery with potent adjuvants can potentially reduce immunization doses, shorten the time of therapy, and induce longer-lived responses. Our data also confirm that serum NicAb titer positively correlates with a blockade efficacy on nicotine entry into the brain, as reflected by an inverse relationship between a brain and serum nicotine level after challenge (figure 5A), consistent with previous investigations [24]. Accordingly, the presence of LVA, MPL or MPL/CpG adjuvant stimulated a higher NicAb titer, and blocked nicotine entry into the brain and retained nicotine in the circulation more efficiently than ID immunization alone (figure 4).

Hypodermic needles were used to delivery nicotine vaccine into the skin in the current study, which has not been commonly adopted for clinical use due to the technical difficulty of this approach. Yet, with the progress made in the past decade in the development of novel technologies for convenient cutaneous immunization [25, 26], intradermal delivery of nicotine vaccine can be readily achieved in the clinic. Currently, ID microinjection systems have been approved by FDA to deliver a reduced dose of seasonal influenza vaccine in adults at 18~64 years of age [27]. LVA can be conveniently combined with this ID microinjection system to improve nicotine vaccine immunogenicity without a modification of vaccine manufacturing. A combination of MPL or MPL/CpG with ID microinjection system may require some modification of vaccine manufacturing but it is practically feasible. Other techniques, like microneedles and transdermal patches, are under development for convenient skin immunization [28, 29]. A combination of LVA, MPL, or MPL/CpG with these delivery strategies can potentially improve nicotine vaccine immunogenicity and thus merits future investigation.

In addition to the modification of a delivery route and incorporation of adjuvants, other strategies have been also evaluated for enhancing nicotine vaccine immunogenicity, like a modification of nicotine conjugation sites, linkers, and carrier proteins, and a combination of

two nicotine vaccines or bivalent vaccines [30-32]. Our strategy can combine with any of these strategies to further improve nicotine vaccine efficacy in the clinic. Moreover, this approach is likely to be effective as well with other similarly designed drug addiction conjugate vaccines against cocaine and opioids (heroin, morphine and oxycodone vaccines).

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Abbreviations

alum	aluminum salt-based adjuvant
ANOVA	analysis of variance
APC	antigen-presenting cell
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
ID	intradermal
IM	intramuscular
LVA	laser vaccine adjuvant
MPL	monophosphoryl lipid A
NicAb	antibody directed against nicotine
Nic-BSA and Nic-KLH	6-carboxymethylureido nicotine conjugated to bovine serum albumin and Keyhole Limpet Hemocyanin, respectively
OVA	ovalbumin
PBS	phosphate-buffered saline
R837	Imiquimod
rEPA	recombinant <i>P. aeruginosa</i> Exoprotein A
TLR	toll-like receptor

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Highlights

Intradermal delivery increases nicotine vaccine immunogenicity.
Incorporation of safe adjuvants further increases nicotine vaccine immunogenicity.
Peak anti-nicotine antibody titer persists longer in adjuvant-incorporated groups.
Intradermal delivery plus adjuvants more efficiently prevents brain nicotine entry.
We present a novel strategy to improve nicotine vaccine immunogenicity.

Figure 1. Superiority of ID over IM nicotine vaccine immunization

BALB/c mice were intradermally (rectangle) or intramuscularly (triangle) immunized with the indicated Nic-KLH doses only once without adjuvants. Two weeks later, serum NicAb titer was measured by ELISA. n=5. **, P<0.01

Figure 2. Reactogenicity of various vaccine adjuvants after ID injection

Low back skin of BALB/c mice was treated with the LVA, TLR agonist-based adjuvants (MPL, R837, CpG), depot adjuvants (Alum, Montanide ISA 720), and combinatorial adjuvants (LVA/MPL, LVA/CpG, MPL/CpG, MPL/R837, MPL/Alum). Five days later, pictures of adjuvant-treated sites were taken (upper panel; scale bar, 2 mm), followed by histological examination (lower panel; scale bar, 100 μ m). The lesion in the skin is outlined by a circle in the upper panel. Arrows in the lower panel in Montanide ISA 720-injected skin point to void bulbs preoccupied by the water-in-oil adjuvant.

Figure 3. Synergistic effects of MPL with LVA or CpG

A. BALB/c mice were ID injected with OVA or MPL-OVA with or without laser pre-illumination. Serum OVA-specific IgG titer was analyzed three weeks after the single immunization. **B.** BALB/c mice were ID injected with Nic-KLH or MPL-Nic with or without laser pre-illumination, or ID injected with MPL/CpG admixed Nic-KLH. Serum NicAb titer was measured two weeks after the single immunization. $n=5$. *, **, ***: $P<0.05$, 0.01, 0.001, respectively.

Figure 4. Superior ID nicotine vaccine immunization with various adjuvants

A. BALB/c mice were IM immunized with Nic-KLH alone (filled triangle) or in combination with Alum (circle), or ID immunized with Nic-KLH alone (filled rectangle) or in combination with LVA (diamond), MPL (triangle), and MPL/CpG (rectangle). Number of immunizations is shown in the parentheses behind the group name. Serum NicAb titer was measured one day before each immunization and 2 and 13 weeks after the last immunization. NicAb titer in the Alum+IM group was compared with the IM group, while NicAb titers in LVA+ID, MPL+ID, and MPL/CpG+ID group were compared with the ID group. **B-C.** Brain and blood nicotine levels following nicotine challenge. Non-immunized or nicotine vaccine-immunized mice were intravenously challenged with 0.01 mg/kg

nicotine and 5 minutes later, brain and blood were harvested and brain (**B**) and serum nicotine levels (**C**) were quantified by gas chromatography. Two-tailed student t-test was used to compare difference between various immunization groups and non-immunized control group or between LVA-, MPL-, and MPL/CpG-incorporated ID immunization group and ID immunization alone in **B**. One-tailed Mann-Whitney test were used to compare difference between LVA-, MPL-, and MPL/CpG-incorporated ID immunization and ID immunization alone in **C**. n=4-6. *, **, ***, p<0.05, 0.01, and 0.001, respectively

Figure 5. Correlation among NicAb titer, brain and serum nicotine level

Correlation between brain and serum nicotine level (A), NicAb titer and brain (B) or serum nicotine level (C) was analyzed by combining data from all the groups together.

Table 1

Peak NicAb titer and duration in various immunization groups

	Immunization times		NicAb titer ($\times 10^3$)	Peak		Peak duration (weeks)
				Fold increase	p value [†]	
Control	None	0.02 $\pm$ 0.00			-	-
IM	7	10.1 $\pm$ 2.7	1	-	0.0012	11
Alum+IM	4	20.3 $\pm$ 5.0	2	0.0318	0.0004	17
ID	3	14.3 $\pm$ 2.3	1.4	0.283	0.0005	19
LVA+ID	3	38.2 $\pm$ 5.1	4	0.0028	0.0003	>19
MPL+ID	2	71.7 $\pm$ 8.6	7	5.68 $\times 10^{-5}$	3.55 $\times 10^{-5}$	>21
MPL/CpG+ID	2	140.6 $\pm$ 19.3	14	1.55 $\times 10^{-5}$	1.30 $\times 10^{-5}$	>21

Values of NicAb titer were expressed as Means $\pm$ SEM (standard error of the mean).

[†]P value was measured by student t-test and compared with IM group.

[‡]P value was measured by student t-test and compared with non-immunized control group.