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Optimization of a methamphetamine conjugate vaccine for antibody production in mice

Misty W. Stevens^a, Melinda G. Gunnell^b, Rachel Tawney^{a,b}, and S. Michael Owens^{a,b,*}

Misty W. Stevens: misty.stevens@intervexion.com; Melinda G. Gunnell: mgunnell@uams.edu; Rachel Tawney: r_tawney@hotmail.com; S. Michael Owens: mowens@uams.edu

^aInterveXion Therapeutics, LLC, 4301 W. Markham St., #831, Little Rock, AR 72205, USA

^bDepartment of Pharmacology and Toxicology, College of Medicine, University of Arkansas for Medical Sciences, 4301 W. Markham St., #611, Little Rock, AR 72205, USA

Abstract

There are still no approved medications for treating patients who abuse methamphetamine. Active vaccines for treating abuse of nicotine and cocaine are in clinical studies, but have not proven effective seemingly due to inadequate anti-drug antibody production. The current studies aimed to optimize the composition, adjuvant and route of administration of a methamphetamine conjugate vaccine, IC_{KLH}-SMO9, in mice with the goal of generating significantly higher antibody levels. A range of hapten epitope densities were compared, as were the adjuvants Alhydrogel and a new Toll-like receptor 4 (TLR4) agonist called GLA-SE. While methamphetamine hapten density did not strongly affect the antibody response, the adjuvant did. Glucopyranosyl lipid A in a stable oil-in-water emulsion (GLA-SE) produced much higher levels of antibody in response to immunization compared with Alhydrogel; immunization with GLA-SE also produced antibodies with higher affinities for methamphetamine. GLA-SE has been used in human studies of vaccines for influenza among others and like some other clinical TLR4 agonists, it is safe and elicits a strong immune response. GLA-SE adjuvanted vaccines are typically administered by intramuscular injection and this also proved effective in these mouse studies. Clinical studies of the IC_{KLH}-SMO9 methamphetamine vaccine adjuvanted with GLA-SE have the potential for demonstrating efficacy by generating much higher levels of antibody than substance abuse vaccines that have unsuccessfully used aluminum-based adjuvants.

Keywords

Methamphetamine; Drug abuse; Therapeutic vaccine; Hapten Density; Adjuvant; Preclinical study

*Corresponding author: phone: 501-686-5487, fax: 501-526-4618.

Conflict of Interest

SMO is a full time faculty member at the University of Arkansas for Medical Sciences. He has financial interests in and serves as Chief Scientific Officer for Intervexion Therapeutics, LLC (Little Rock, AR), a pharmaceutical biotechnology company focused on treating human drug addiction with antibody-based therapy.

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1. Introduction

There remains an unmet need for medications to treat and prevent the serious consequences of methamphetamine (METH) abuse and addiction. A potential solution to this problem is the use of immunotherapy to sequester the METH in the bloodstream and restrict its entry into the brain. Vaccines against METH have been developed and are in various stages of testing in multiple labs [1–8]; none yet has reached the clinic. A monoclonal antibody against METH has been developed and tested in a Phase Ia clinical study [9], but efficacy studies are still in the future. The monoclonal route allows administration of high levels of antibodies and provides immediate therapy. But for some patients, who are able to mount a high and sustained immune response, an active vaccine may be a viable, and cheaper, alternate.

In clinical studies of vaccines for cocaine and nicotine, the most common reason for failure has been a lack of effective antibody titer generation in a majority of subjects [10]. Unfortunately, an adequate antibody response is a primary requirement for efficacy [11]. Adjuvants are typically used to increase the immune system's response to a vaccine, however very few adjuvants have been approved as part of human vaccine products. The majority of approved vaccines use one of the aluminum-based adjuvants, such as Alhydrogel, grouped under the name Alum. This is also the adjuvant used for the cocaine and nicotine vaccines previously tested [12,13].

More recently, adjuvants based on lipopolysaccharide (LPS) that are Toll-like receptor 4 (TLR4) agonists have been developed and used in human studies (reviewed in [14]). GlaxoSmithKline has gained approval for two vaccines that contain monophosphoryl lipid A (MPL, a detoxified version of LPS) adsorbed on to aluminum, called AS04 (Cervarix prescribing information). Other vaccine formulations containing MPL are currently in clinical trials. Lockner et al. used liposomes containing MPL in preclinical studies of a nicotine vaccine and concluded that TLR2 and/or TLR4 agonism was necessary for a sufficient antibody response [15]. Pushing the field even further, Immune Design Corp has developed/licensed a purely synthetic Lipid A analog called glucopyranoside lipid A (GLA) which retains potent immunostimulatory characteristics, but has significantly reduced toxicity. Importantly, GLA's synthetic origin adds manufacturing advantages such as safety and homogeneity [16]. GLA is a clinical stage adjuvant being used in multiple Immune Design and partnered vaccine programs (Immune Design website and [17,18]). When GLA is administered in an oil-in-water stable emulsion (GLA-SE), the antibody response is enhanced [14,19,20].

We have developed a METH conjugate vaccine, called IC_{KLH}-SMO9, and performed preliminary safety/efficacy studies. Rats immunized with IC_{KLH}-SMO9 adjuvanted with Alhydrogel produced antibodies that sequestered METH in the blood, and were able to maintain normal levels of food consumption after high dose METH challenges [6]. To increase the levels of antibody production and prepare for human trials, better adjuvants like GLA-SE should be tested. The objective of these current studies was to optimize the formulation of IC_{KLH}-SMO9 and details of its administration in mice prior to starting additional efficacy studies in rats. To accomplish this objective, we evaluated hapten epitope

densities, adjuvants, and routes of administration and compared the antibody responses by concentration and METH-binding capacity over time.

2. Materials and methods

2.1. Drugs and reagents

(+)-Methamphetamine hydrochloride (METH) and the tritiated form of METH ($[^3\text{H}]$ -METH) were obtained from the NIDA Drug Supply Program. Alhydrogel was from Brenntag Biosector (Frederikssund, Denmark), Sigma Adjuvant System[®] (SAS) was from Sigma Aldrich (St. Louis, MO), and GLA-SE adjuvant was from Immune Design Corp. (Seattle, WA). Sulfo-SMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate) crosslinker and TCEP-HCl (Tris(2-carboxyethyl)phosphine hydrochloride) were purchased from Thermo Fisher Scientific Inc. (Pierce, Rockford, IL). Immunocyanin, two purified monomers of keyhole limpet hemocyanin (KLH) called IC_{KLH}, was obtained from Biosyn Corp. (Carlsbad, CA).

2.2. Vaccine production

All vaccines were made by conjugating a METH-like hapten (SMO9) to immunocyanin to make the METH-conjugate vaccine called IC_{KLH}-SMO9. The procedure was similar to the method of Peterson et al. [21]. In brief, IC_{KLH} was activated by the addition of Sulfo-SMCC crosslinker, then desalted to remove unused linker. Separately, the METH-like hapten dimer was reduced to a monomer using TCEP-HCl. Reduced hapten was added to activated IC_{KLH} and stirred for two hours to allow the conjugation to occur. Finally, the conjugated material was mixed with excess reduced cysteine to cap any remaining activation sites. All reactions were performed with an overlay of Argon gas. The final product was dialyzed into 50 mM sodium phosphate, pH 7.3 with 5% lactose.

Separate conjugations resulted in three lots of vaccine with hapten densities of 13, 16, and 23 SMO9 molecules per KLH monomer as measured by the method reported in Peterson et al. [21]. These are labeled IC_{KLH}-SMO9₁₃, IC_{KLH}-SMO9₁₆, and IC_{KLH}-SMO9₂₃, respectively.

2.3. Immunization and sampling protocols

Female Balb/c mice were purchased from Charles River Laboratories and immunized by subcutaneous (SC) or intramuscular (IM) injection with 20 μg of IC_{KLH}-SMO9 in one of three adjuvants as described in each experiment (Table 1). Booster injections were administered at 3 and 9 weeks with an additional injection given at week 15 to groups 1–4. All boosts were replicates of the original immunization. Eight (8) animals were assigned to each group; one mouse in group 3 was sacrificed in the 23rd week due to illness.

Blood samples were taken by tail cut and allowed to clot for 1 hour at room temperature; trunk blood was collected at termination. Following centrifugation at $14,000 \times g$ for 10 min, the serum was separated and stored at -30°C until thawed for analysis.

All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences.

2.4. Characterization of the antibody response

2.4.1. Interpolated titer ELISA—The relative concentration of anti-METH polyclonal antibodies in serum samples was determined by an interpolated titer ELISA using a mixture of 6 anti-METH monoclonal antibodies produced in house as the standard. First, 96-well plates were coated with 0.25 µg/mL ovalbumin crosslinked with a METH-like hapten. Second, plates were blocked with SuperBlock (Pierce Biotechnology, Inc.). Third, mouse serum samples, standard curve, quality control, and blank samples were added to wells of each plate in duplicate. Fourth, the detection reagent, goat-anti-mouse (Fc-specific) antibody labeled with alkaline phosphatase, was added at 1 µg/mL. Each step after coating was incubated at room temperature for 1 hour with shaking on a plate shaker. Between steps 2 through 4, the plate was washed with phosphate-buffered saline containing 0.1% Tween 20 (wash buffer) to remove unbound material. Finally, chromogenic substrate solution was added to the wells, the plate was incubated at 37°C for 30 minutes and then read with a plate reader.

Each test sample was diluted 1:4000 in 50% SuperBlock in wash buffer and with naïve mouse serum added so that the final concentration was 1%. The standard curve, quality control samples and blanks were prepared in the same buffer with 1% serum. Precision and accuracy acceptance criteria were met for all reported data. Reported concentrations are relative to the specific standard curve used and should only be used for the comparisons herein.

2.4.2. Equilibrium dialysis to quantify antibody binding to METH—As described in Rüedi-Bettschen et al. [6], serum samples from each mouse were diluted 1:100 or 1:300 in buffer and mixed with ³H-METH (5 nM final) before being added to one chamber of an insert in a rapid equilibrium dialysis device (Pierce, Thermo Scientific, Rockville, IL). Buffer only was added to the other chamber and the entire plate was incubated overnight at 4°C to equilibrate. The labeled METH in each chamber was measured to determine the amount of ³H-METH bound by the test serum.

2.4.3. Radioimmunoassay to determine dissociation constants—A published bead-based radioimmunoassay [22] was used to determine the METH-binding K_d values for serum samples at selected timepoints.

2.4.4. Statistical Analysis—Antibody concentrations and percent ³H-METH binding values (1:100 dilution) were compared by two-way repeated measures analysis of variance (day x treatment group), followed by Bonferroni Multiple Comparison tests (Figures 1–3 and 5). The average affinity of antibodies generated in groups 4 and 6 at week 11 were found to be normally distributed by a Shapiro-Wilk test and therefore compared by an unpaired t-test (Figure 4). Statistical analysis was performed with GraphPad Prism software version 6.0 (La Jolla, CA). Differences were considered to be statistically significant at $p < 0.05$.

3. Results

Two experiments were conducted with the goal of optimizing the METH-conjugate vaccine, IC_{KLH}-SMO9. The first optimized vaccine hapten density while the second focused on adjuvants. The experimental design is summarized in Table 1. Groups 1–4 were completed prior to initiation of groups 5–7. Animals for study session 1 were purchased in a single batch; animals for session 2 were purchased in a second batch.

3.1. Comparison of hapten densities

The first study compared vaccines with different hapten densities (groups 1–3); all were administered with SAS as adjuvant given by SC injection. Like GLA-SE, SAS is a TLR4 agonist that contains monophosphoryl lipid A in a stable oil-in-water emulsion. However, unlike GLA-SE, which is a clinical stage synthetic compound, SAS is isolated from *Salmonella Minnesota* and is only approved for animal use. As shown in Figure 1, there were no significant differences in elicited antibody concentration or METH binding capacity among groups (as determined by overlapping standard deviations), but the greatest average antibody concentration was elicited by the highest hapten density MCV, IC_{KLH}-SMO9₂₃, with 23 haptens per KLH molecule (top panel, group 3).

Both antibody concentration and METH binding function increased in all groups after the second boost at 9 weeks, but no further increase was observed after the 3rd boost at 15 weeks (Figure 1). In fact, a slight decrease in the antibody concentration, but not function, was seen by week 33. This is hypothesized to result from affinity maturation whereby the lower affinity antibodies cease to be produced, resulting in lower total antibody concentrations. But, because the higher affinity antibodies are still present, the METH binding function is retained.

Animals in group 4 were also immunized by SC injection with IC_{KLH}-SMO9₂₃, but it was administered with Alhydrogel to demonstrate the higher antibody concentrations generated by the TLR4 agonist adjuvants. Following the first boost at 3 weeks, the Alhydrogel-induced antibody concentrations were near the SAS results (~100 µg/mL), but subsequent boosts did not produce a significant increase and the relative antibody levels remained steady just below 200 µg/mL through week 33 (data not shown). At all timepoints, the METH binding capacity of the serum from Alhydrogel immunized animals was lower than that from the SAS immunized animals.

3.2. Increased responses from improved adjuvants

Using IC_{KLH}-SMO9₂₃, three groups of mice (groups 5–7) were immunized in the second study using different adjuvants: SAS and GLA-SE at two concentrations, 1 or 5 µg. Immune Design Corp. recommends that their GLA-SE adjuvant be administered by intramuscular injection, therefore the comparator immunization with SAS was also given by IM injection.

Average antibody concentrations at each time point are graphed in Figure 2 (upper panel). The highest amounts of antibody were generated from the GLA-SE adjuvant, with 5 µg GLA-SE being somewhat more productive than 1 µg GLA-SE. Both GLA-SE and SAS showed definite boost effects in serum at 5 and 11 weeks following the immunizations at

weeks 3 and 9. In addition, an immunization given at week 37 to the 5 μ g GLA-SE group resulted in a modest increase in antibody concentration two weeks later (data not shown).

The average METH binding function of the immune response generally follows the shape of the concentration-time graph (Figure 2, lower panel), however, the correlation between individual animal antibody concentration and function results was best (~50%) at time points immediately following boosts (weeks 5 and 11). Later, the results were less correlated, again suggesting that as affinity maturation occurs differently in each animal, less antibody may be needed to retain function. For instance, at week 33, the average antibody concentration was approximately the same as week 5, but the METH binding capacity remained higher at the later time point.

An additional comparison was made between the two clinical adjuvants, Alhydrogel and GLA-SE. In addition to the quantity of antibody made being much larger by GLA-SE adjuvanted vaccinations (data not shown), the capacity for binding METH was significantly greater at all measured points after the first boost (Figure 3). Even the third boost at week 15 for the Alhydrogel group did not affect the results.

3.3. Affinity of antibody response by adjuvant

Selected time points from group 6 which received the IC_{KLH}-SMO9₂₃ vaccine with 5 μ g GLA-SE, from group 3 which got IC_{KLH}-SMO9₂₃ vaccine with SAS, and from group 4 which got IC_{KLH}-SMO9₂₃ vaccine with Alhydrogel were analyzed by radioimmunoassay to observe the affinity maturation over time. When mixtures of IgG are analyzed by this method, the K_D (dissociation constant for binding METH) of the antibody with the highest affinity (lowest K_D value) is observed. As shown in Table 2, the average of affinities measured from the 5 μ g GLA-SE group increases after the first two boosts at weeks 3 and 9. By week 11, the affinity seems to have matured to a stable level.

At week 11 the variability between animals is much larger when Alhydrogel is used. Figure 4 shows the affinity results from week 11 comparing the responses from animals receiving the vaccine adjuvanted with Alhydrogel subcutaneously (group 4) versus 5 μ g GLA-SE intramuscularly (group 6). These two clinically used adjuvants yield antibodies with significantly different affinities for METH, with GLA-SE producing a higher affinity and more reproducible antibody response.

3.4. Route of administration affects early response levels

From these collected data, a comparison was made of the effects of route of administration on immunizations with IC_{KLH}-SMO9₂₃ and SAS as adjuvant. As shown in Figure 5, intramuscular (IM) injections (group 7) elicit higher average antibody concentrations following initial and second boosts (weeks 3 and 9) compared to subcutaneous (SC) injections (group 3). The immunization protocols differed after 11 weeks, so no further comparisons were made.

4. Discussion

The vaccine optimization studies presented here were conducted in mice to select a lead candidate vaccine, adjuvant, and route of administration for further testing. As shown by the comparisons of vaccines with different hapten densities, the range of densities tested between 13 and 23 haptens per KLH monomer did not show dramatic differences, suggesting that vaccine batches made within this range may be similarly effective.

Importantly, the TLR4 agonist adjuvants, SAS and GLA-SE, generated much stronger antibody responses than the commonly used Alhydrogel adjuvant. Comparisons between SAS and Alhydrogel adjuvanted vaccines given by SC injection demonstrated an approximately 50% increase in response from SAS over Alhydrogel. When SAS was compared to GLA-SE with vaccines given by IM injection, the GLA-SE produced about twice the amount of antibody made by SAS. Importantly, these high antibody levels were sustained without boosting for a long time, remaining above 600 µg/mL from week 11 through the end of the measured period at week 33 (Figure 2). Though not absolutely comparable due to the differences in quantitation methods, the SAS adjuvanted METH vaccines tested by Moreno et al. generated antibody concentrations of 150–220 µg/mL [1]. Further, a nicotine vaccine that later entered clinical trials generated <200 µg/mL in rats when administered with the highly potent Freund's adjuvant that is not allowed in human studies [23]. Together, these data strongly suggest that GLA-SE adjuvanted IC_{KLH}-SMO9 should stimulate higher levels of anti-METH antibodies in human subjects than Alhydrogel. If antibody levels are indeed a significant factor for vaccine efficacy, as measured by the capacity to reduce relapse rates to drug use, this combination of IC_{KLH}-SMO9 with GLA-SE may be more successful.

While the comparisons between adjuvants showed distinct differences in antibody levels produced, the resulting antibody affinities were not as affected. It is hypothesized that the hapten-carrier combination and the immunization schedule have the greatest effect on affinity while the adjuvant has the greatest effect on concentration; these data support that conclusion.

In preparation for FDA regulated studies, plans have been made for larger-scale production of toxicology and clinical batches of the MCV. Dose-range finding and Good Laboratory Practice compliant toxicology studies will be conducted prior to a Phase 1 safety study in healthy volunteers. Since we have now optimized the MCV's chemical composition and selected a superior adjuvant, this product has great potential to result in a safe human vaccine suitable for reducing METH's adverse effects while helping the patient withstand the challenges of binges and relapse, which can lead to recidivism during treatment programs.

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responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations

GLA	glucopyranosyl lipid A
IC_{KLH}	immunocyanin monomers of KLH
IM	intramuscular
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
MCV	METH-conjugate vaccine
METH	(+)-methamphetamine
SMO9	METH-like hapten (S)-N-(2-(mercaptoethyl)-6-(3-(2-(methylamino)propyl)phenoxy)hexanamide
MPL	monophosphoryl lipid A
RED	rapid equilibrium dialysis
SAS	Sigma adjuvant system
SC	subcutaneous
SD	standard deviation
SE	stable emulsion
TLR4	Toll-like receptor 4

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Highlights

- METH-conjugate vaccine produces high affinity antibody response in mice
- The range of hapten densities tested did not greatly affect the antibody response
- GLA-SE adjuvant produces higher antibody levels than other tested adjuvants
- Adjuvants like GLA-SE may be a key to effective human substance abuse vaccines

Figure 1. Average relative antibody concentration (upper panel) and function (% $^3\text{H-METH}$ bound, lower panel) + or - SD over time from MCVs with different hapten densities and SAS as the adjuvant

Arrows indicate the timing of repeated immunizations. Data points are slightly offset and error bars are a mix of plus and minus the mean for visual clarity, but samples were taken on the same date. A two-way repeated measures analysis of variance (day x treatment group), followed by a Bonferroni Multiple Comparison was used for statistical comparison; no differences were noted.

Figure 2. Average relative antibody concentration (upper panel) and function (lower panel) + or – SD over time elicited by different adjuvants

All groups were immunized with MCV with 23 haptens per KLH molecule, administered with the adjuvant as labeled. Black arrows indicate boosts for all groups. Data points are slightly offset for the 1 µg GLA-SE group for visual clarity. A two-way repeated measures analysis of variance (day x treatment group), followed by a Bonferroni Multiple Comparison was used for statistical comparison; *p<0.05 for 5 µg GLA-SE vs SAS, **p<0.05 for 1 µg GLA-SE vs SAS.

Figure 3. Average METH binding function over time by clinical adjuvants

Both groups were immunized with MCV with 23 haptens per KLH molecule, administered with the adjuvant as labeled. Solid arrows indicate boosts for both groups with the dashed arrow for the Alhydrogel group only. A two-way repeated measures analysis of variance (day x treatment group), followed by a Bonferroni Multiple Comparison was used for statistical comparison; * $p < 0.05$.

Figure 4. Average antibody affinity at week 11

The measured affinity for METH in serum samples from week 11 is graphed from individual mice with mean and SD bars shown. An unpaired t-test was used for statistical comparison; * $p < 0.05$.

Figure 5. Average relative serum antibody concentration + SD produced by immunization by different routes of administration

Mice were given IC_{KLH}-SMO9₂₃ with SAS by intramuscular (IM) or subcutaneous (SC) injection. Boosts were given two weeks prior to each analysis time point. A two-way repeated measures analysis of variance (day x treatment group), followed by a Bonferroni Multiple Comparison was used for statistical comparison; *p<0.05 for IM vs SC.

Table 1

Experimental Design

Study Session	Study Group	Adjuvant	Immunization Schedule (weeks)	Route of Administration	Hapten Density
1	1	SAS	0/3/9/15	SC	13
	2	SAS	0/3/9/15	SC	16
	3	SAS	0/3/9/15	SC	23
	4	Alhydrogel	0/3/9/15	SC	23
2	5	1 µg GLA-SE	0/3/9	IM	23
	6	5 µg GLA-SE	0/3/9	IM	23
	7	SAS	0/3/9	IM	23

Table 2

Dissociation constant values from test serums at selected weeks

Week	K_D (nM ± SD) from IC_{KLH}-SMO9₂₃		
	5 µg GLA-SE (IM)	SAS (SC)	Alhydrogel (SC)
5	41 ± 11.3	-	-
9	28 ± 5.4	-	-
11 ^a	13 ± 2.0	15 ± 3.7	22 ± 8.3
15	17 ± 3.2	-	-
17	-	14 ± 3.3	20 ± 8.3
21	13 ± 2.6	-	

Note: Where marked with a dash, samples were not analyzed at that time point. SD, standard deviation.

^aSee Figure 4 for statistical comparison of GLA-SE and Alhydrogel affinities at week 11.