A novel bivalent morphine/heroin vaccine that prevents relapse to heroin addiction in rodents

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Abstract

Both pre-clinical and clinical studies make feasible the use of vaccines as novel therapeutic medications to treat drug addiction. No reports to date have shown the development of structural models of opiate candidate vaccines for treating human addiction to such compounds. Here we report on the initial development of a novel structural formulation of a bi-valent vaccine against morphine/heroin. This vaccine was able to trigger and establish a high titer antibody response to haptenized drug with antibodies displaying equivalent specificities for both morphine and heroin. Such antibodies did not cross-recognize structurally dissimilar opiate medications. Furthermore, the evaluation of the potential therapeutic effectiveness of this vaccine was targeted to relapse prevention using a schedule of heroin delivery in the rat self-administration model. Antibodies against heroin blocked its reinforcing effects in rodents. The type of carrier protein used in this vaccine allows further evaluation of its potential therapeutic value for preventing relapse to heroin addiction in humans.
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1. Introduction

Heroin addiction is a health problem and successful pharmacotherapies have been developed to treat the detoxification, withdrawal, craving and maintenance of the abstinent state of this addictive disorder [1–6]. Such treatments use long-acting oral opiate agents such as methadone, levo-α-acetylmethadol (LAAM) and the recently FDA-approved buprenorphine that mimic drug action at the opioid receptor [6–8]. Although the effectiveness of drug mimicry has been documented clinically, there remains significant concern and skepticism towards such treatments [2]. This relates to the fact that the heroin addict is still being exposed to opiates, and consequently may develop tolerance to and experience withdrawal from the treatment agent [2]. Another problem often seen in most out-patient treatments during the mainte-

nance of heroin abstinence is the high incidence of relapse to addictive drug consumption [1–6]. An alternative pharmacotherapy for both abstinence and prevention of relapse to heroin addiction is based on blocking heroin from reaching its opioid receptor target by using naltrexone and naloxone [7]. Indeed, despite the fact that these antagonists can be used to treat heroin addiction, their use has limitations as both drugs are not inactive in the absence of exogenous opiates and they block the binding of the body's endogenous opioids (e.g., enkephalins and endorphins) causing negative emotional effects in the long-term treated patient [2].

As for other drugs of abuse such as cocaine, nicotine, methamphetamine and phencyclidine [9–11, and references therein], an alternative approach for heroin addiction might be an antibody-based antagonism of heroin's brain entry. The feasibility of using an active vaccination approach for treating heroin addiction was explored 30 years ago by Bonese et al. [12]. They showed that responding maintained by this opiate was selectively abolished after active immunization with a morphine-6-hemisuccinyl-bovine serum albumin (BSA)

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(M-6-H-BSA) conjugate in a single rhesus monkey trained to self-administer heroin. Surprisingly, no further progress was made in conducting more systematic studies focused on the generation of non BSA-based carrier protein models of opiate vaccines appropriate for human use. Besides, we propose that once validated at the pre-clinical level, a novel structural model of a heroin vaccine designed for clinical use, could be subsequently considered as a candidate medication in clinical trials to test its potential therapeutic effectiveness as a co-adjuvant and synergistic therapy for methadone or buprenorphine in preventing relapse to this opiate addiction. Furthermore, the use of such a vaccine in combination with existing therapies could also be useful to reduce the duration of substituted addiction and development of toxic side-effects [13] often seen with the long-term use of these pharmacological agents, that ultimately contribute to the high incidence of treatment abandon and heroin relapse [13].

2. Materials and methods

2.1. Preparation of the morphine-tetanus toxoid (M-TT) vaccine

The synthesis of the hapten for M-TT vaccine used the commercial formulation of morphine-sulfate·5H$_2$O (m.w. 758.8) (Sigma–Aldrich, St. Louis, MO, USA) as initial compound to produce morphine base according to a standard experimental procedure reported by Simon et al. [14]. After synthesis, morphine base was subjected to selective succinylation of its 6-hydroxy group in reflux (6 h at 70–80 °C) according to a standard protocol initially reported by Wainer et al. [15,16] with an excess of succinic anhydride (Sigma–Aldrich, St. Louis, MO, USA) (2 g per 1 g of morphine sulfate) in dry benzene or pyridine (Sigma–Aldrich, St. Louis, MO, USA). The residue forming the intermediate derivative named morphine-6-hemisuccinyl (M-6-H) was rinsed and re-crystallized ten consecutive times with 60% ethanol/40% distilled H$_2$O (v:v). Thin layer chromatography (TLC) was performed in a solvent system of ethanol/dioxan/benzene/NH$_4$OH (8:1:10:1, v:v:v:v) in order to determine the yield of M-6-H. The product, synthesized and crystallized as described above, usually contained less than 5% of morphine base. Analyses of the UV spectrum of M-6-H demonstrated undistinguishable values from that of morphine, in which the chromophore responsible for the peak at 285 nm is the phenolic ring, a clear evidence for the absence of reaction at this site (e.g., the 3-hydroxy group of the phenolic ring of morphine). One hundred milligrams of M-6-H derivative were activated for coupling in 100 ml of distilled H$_2$O in the presence of 100 mg of water-soluble 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, Pierce, Rockford, IL, USA). The pH of the mixture was adjusted to 5.5 with 1N HCl. After 2 h at 37 °C of incubation of the reaction mixture, titration of M-6-H with NaOH was performed to determine titratable car-

boxy groups at a pKa of 4.3 potentially present in non-reacted free succinyl residues esterified to the 6-hydroxy group of morphine base. These measurements usually showed yields of ≈98% of the EDC-M-6-H derivative. This latter compound is usually unstable in aqueous solutions and should be promptly reacted with the tetanus toxoid (TT) used as carrier protein (CP). The TT (which was the recombinant non-toxic polypeptide H chain of the tetanus toxin, with an approximate molecular mass of 100 kDa and 98% of purity) was covalently derivatized with the amine-reactive cross-linker reagent with a latent amino group available on demand the *N*-(ε-trifluoroacetylcaproyloxy)succinimide ester (TFCS, Pierce, Rockford, IL, USA) 24 h before coupling with the EDC-M-6-H intermediate. The TFCS was coupled by its NHS ester functional group to the ε-amino groups of the side chain of lysine residues of the TT. In a representative synthesis, a 20-fold molar excess of TFCS (134 mM) is dissolved in a freshly prepared solution of 10–20% DMSO (Sigma–Aldrich, St. Louis, MO, USA)/80% distilled H$_2$O and 50 μl of this solution is mixed with 0.5 mM of TT in a volume of 4 ml of PBS, pH 7.2. Thereafter, the reaction mixture was incubated at room temperature overnight and the trifluoroacetyl protecting group of the coupled TFCS is removed by incubation at pH 8.1 in PBS at room temperature for up to 3 h. The final purification of the TT-TFCS derivative was carried out by exhaustive dialysis against PBS, pH 7.2. To prepare the conjugate, the EDC-M-6-H derivative was coupled by its free imide group to the deprotected primary amine group of the TT-TFCS derivative. In a representative synthesis, 1 μmol of TT-TFCS (e.g., ≈0.7 μmol of active sites for covalent condensation) was mixed with 30 mg of the EDC-M-6-H derivative (≈70 μmol of active sites for covalent condensation) in a volume of 100 ml of PBS, pH 7.5 and the reaction mixture was incubated at room temperature overnight in gently stirring. After exhaustive dialysis against PBS, pH 7.4, the purified conjugate was concentrated by pressure dialysis, aliquoted (unit dose = 1 mg of TT/ml), freeze-dried, lyophilized and stored in sealed sterile glass vials. As a control used as antigen for the solid phase of ELISA assays (see below), a synthesis of an additional conjugate was carried out as above where BSA (Sigma–Aldrich, St. Louis, MO, USA) replaced TT as carrier protein. The percentage of conjugation of hapten to carrier protein TT or BSA was determined by titration of the number of free amines (lysine residues) using *o*-phtaldehyde (Sigma–Aldrich, St. Louis, MO, USA) according to a standard procedure [17]. Generally, the percentage conjugation was between 65 and 85%.

2.2. Active immunization and ELISA assays

The active immunization studies used male adult rats of 250–280 g (Harlan, Mexico). During all experimental conditions they were housed in individual hanging plastic cages within a temperature (21–24 °C) and light (08:00 h on; 20:00 h off) controlled vivarium. Principles of laboratory animal care, as outlined in the National Institutes of

Health (NIH) were followed. Although this protocol was not designed to specifically address toxicity, no detrimental effects of active vaccination were observed in boosted animals. For initial evaluation of the immunogenicity of the M-TT vaccine, 10 rats were immunized with the M-TT vaccine adsorbed to aluminum hydroxide gel adjuvant (alum, Imject-R-Alum, Pierce, Rockford, IL, USA) according to manufacturer's protocol (Pierce, Rockford, IL, USA). Both adjuvant and TT alone were used as controls ($n = 10$ for each group) in the active immunization protocol. Each animal was injected subcutaneously (s.c.) at four sites over the shoulders bilaterally (two inoculation/side), with a total dose of M-TT vaccine/adjuvant of 250–300 µg/kg/0.25–0.3 ml alum ($\approx$72 µg/inoculation/animal/boost). Subsequently, 7–8 booster injections were administered using the same unit dose and coadjuvant and were given over a period of 14–16 weeks (once biweekly). This dosing regimen was adapted from standard vaccination protocols in rodents recommended for elicited antibody titer responses to small haptens linked to high molecular mass carrier proteins [18]. Fourteen days after each boost animals were invariably bled and sera were collected and frozen at −20 °C until use. A solid-phase antibody capture ELISA assay [19] was used for monitoring antibody titer response after each boost in vaccinated animals. Briefly, ELISA plates were coated with M-BSA conjugate to limit antibody detection to morphine and antisera were added at a range of serial dilutions in triplicate. A biotin-labeled secondary anti-rat antibody (Jackson Immunoresearch, West Grove, PA, USA) coupled to a detection system of the VECTASTAIN Elite ABC Peroxidase Kit (Vector Lab, Burlingame, CA, USA) and OPD (Sigma–Aldrich, St. Louis, MO, USA) as chromogenic substrate were used to detect immunopositive signals. Antibody titers were initially defined as the inverse of the serum dilution that gave 50% of the maximal response. No anti-morphine antibodies were detected following immunization with either alum or TT alone (data not shown). A standard equilibrium dialysis assay [20] with radiolabeled morphine (^{14}C-morphine, 57 mCi/mmol, Amersham Bioscience Corp., Piscataway, NJ, USA) was used to quantify the antibody concentration of the immune sera and values were expressed in mg/ml of sera. These data were used to estimate the heroin-binding capacity of the immune serum in mg/ml and the affinity of the antisera for heroin binding. A pool of high-titer antisera ($\approx$1:100,000) contained high-affinity antibodies (2.5–$4 \times 10^9 \, M^{-1}$) with a combined heroin binding capacity of up to 9–14 µM (0–6–0.8 mg/ml antigen-specific antibody). The specificity of the anti-morphine antibodies was determined by using the ELISA assay described above, where antisera from rats immunized with the M-TT vaccine were incubated with varying concentrations of competitors (0.0001–100 µM) represented by heroin and its metabolites (6-monoacetyl-morphine and morphine 3-and-6-glucuronides, Sigma–Aldrich, St. Louis, MO, USA). Additional competitors used included compounds such as naloxone (Sigma–Aldrich, St. Louis, MO, USA), naltrexone (Sigma–Aldrich, St. Louis, MO,

USA), methadone (Sigma–Aldrich, St. Louis, MO, USA) and buprenorphine (Sigma–Aldrich, St. Louis, MO, USA) as well as Leu-enkephalin and β-endorphin (Tocris Cookson, Inc., Ellisville, MO, USA).

2.3. Study of active immunization antagonism on heroin self-administration

Antibody antagonism studies used male adult rats of 250–280 g (Harlan, Mexico). During all experimental conditions they were housed in individual hanging plastic cages within a temperature (21–24 °C) and light (08:00 h on; 20:00 h off) controlled vivarium. Principles of laboratory animal care, as outlined in the National Institutes of Health (NIH) were followed. Heroin self-administration experiments were carried out in eight experimental operant chambers for rats (TSE Systems, Bad Homburg, Germany) fully equipped for self-administration of drugs. The chambers were connected to an OBS interface (TSE Systems, Bad Homburg, Germany), and a Pentium 4 PC was programmed with TSE software (TSE Systems, Bad Homburg, Germany) to control experimental events. Rats were divided into three groups (10 animals per group for a total of 30 animals) for the heroin self-administration paradigm, which used a unit dose of 0.06 mg/0.2 ml of sterile saline solution/kg body weight per infusion heroin (Sigma–Aldrich, St. Louis, MO, USA). Our heroin self-administration protocol in the rat was based on reported protocols [17,21] in which this dosing regimen produces opiate tolerance and dependence that parallels the behavior of chronic heroin use in humans. Cues (i.e., tone plus light) were presented to signal heroin availability during drug self-administration training and were absent along both extinction and reinstatement. Briefly, prior to surgery, rats ($n = 30$) were trained to press a lever for the delivery of food pellets. Jugular vein catheters were then implanted, and 1 week later the heroin self-administering protocol began. In daily 4 h sessions, animals were trained to self-administer intravenous infusions of 0.06 mg/kg heroin on a fixed-ratio 10 schedule of reinforcement (FR10). A minimum of 10 days of stable baseline response rates of ±10% variation in the number of infusions, at the FR10 in all animals was defined as baseline acquisition. After heroin intake had stabilized, substitution of normal saline was used for extinction of heroin self-administration behavior. Animals were normally subjected to an additional cycle of heroin intake acquisition/extinction before beginning immunization. Following heroin self-administration baseline training, sessions were discontinued for a 2-week period, and then rats were immunized with alum alone ($n = 10$), alum plus TT ($n = 10$) and alum plus M-TT vaccine using the immunization regimen described above. During the immunization period, heroin self-administration sessions were discontinued and sera were collected from immunized animals 14 days after each boost for monitoring the antibody titer using the ELISA assay described above. Boosted animals generally acquired sustained maximal antibody titers after the fourth to fifth

boosts. Reacquisition of heroin self-administration behavior was studied with the 0.06 mg/kg dose of heroin in all control and M-TT vaccine-treated rats. This dose was studied for 10–15 post-immunization sessions in all immunized groups. Analyses of antibody effects were calculated by averaging the total number of heroin infusions (drug intake) over the 10–15 post-immunization days in order to provide an overall evaluation of the entire post-immunization effects.

2.4. Statistical analyses

Statistical differences of antibody effects over the 10–15 post-immunization sessions in all immunized groups (control and M-TT vaccinated) were calculated from one dependent measure (e.g., infusions) for between-group treatment comparisons. Two-factor analysis of variance (ANOVA) was performed and the Newman–Keuls procedure was use for post-hoc comparisons. Significance was set as $P \leq 0.05$.

3. Results and discussion

3.1. Vaccine design

We initially designed and synthesized a novel structural formulation of a morphine-tetanus toxoid (M-TT) vaccine able to generate a robust and sustained immunologic humoral response for heroin upon active vaccination. In the M-TT conjugate (patent pending #PCT/MX2005/000049), drug was covalently haptenized to the ε-amino free groups of the side chain of lysine residues of the highly immunogenic carrier protein tetanus toxoid (TT) using a long ($\approx$20.15 Å) spacer linker arm (Fig. 1). Overall, the structural design of this morphine conjugate was based on exploiting practical structural rules derived from previous works, reporting successful preparations to make highly antigenic small hapten-based conjugates [19,22]. There has been demonstrated that the length of the spacer linker arm and its degrees of freedom, place the haptenized moieties of each conju-

gate, distant enough from structural domains of the carrier protein and in appropriate spatial orientation to be recognized by the immune system as antigen [23,24]. This in turn causes such haptens to become highly immunogenic [23]. With this rationale, we first hypothesized that our structural formulation of the M-TT vaccine should be efficient enough for triggering a robust response of antibodies of differing affinities and specificities to different structural determinants of morphine shared with related structural opiate analogs such as heroin and its neuroactive metabolites. Second, the M-TT vaccine not only uses a highly immunogenic carrier protein appropriate and authorized [22] for human use but also introduces a spacer linker arm that is twice the length of ones normally used in other morphine-6-hemisuccinyl-BSA conjugates to covalently haptenize morphine to its C-6 hydroxyl function through two stable amide bonds (Fig. 1). This spacer linker arm is formed by an aliphatic chain of carbon atoms of low structural complexity, which may reduce its potential role as immunopredominant determinant when haptenized morphine is exposed to immune cells.

3.2. Vaccine immunogenicity and characterization of humoral response

We further investigated the immunogenic capability of the M-TT vaccine to trigger a sustained and robust humoral immunologic response to the haptenized drug [12,23,25]. Although this protocol was not designed to specifically address animal toxicity testing, no major detrimental effects at general health level such as anaphylaxis, convulsions, loss of body weight and appetite, as well as in differences in ambulatory activity and mortality were observed between control versus vaccinated animals. Other safety parameters measured included injection site inspection and palpation. However, there were little local injection reactions such as erythema and subcutaneous nodules early (i.e., 1–7 days) after immunization, probably due to mechanical process of M-TT vaccine injection. Nonetheless, further standard
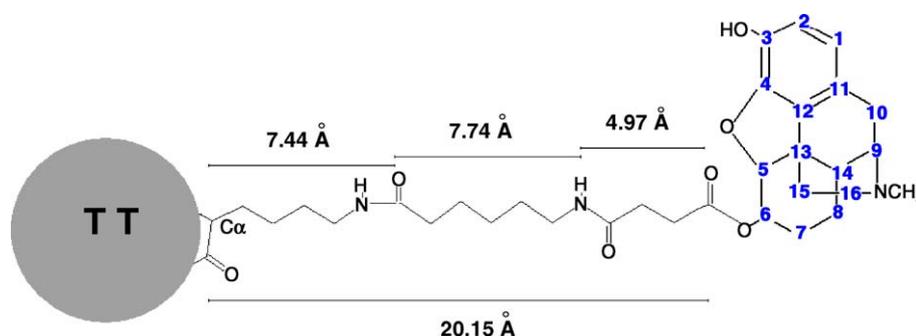

Fig. 1. Structural design of the novel morphine-tetanus toxoid (M-TT) vaccine. The total length of the linker spacer arm is nearly 20 Å and is formed by an aliphatic chain of carbon atoms containing two stable amide bonds. The morphine-6-hemisuccinyl-morphine (M-6-H) was coupled to the reactive intermediate EDC-M-6-H. The ε-amino groups from the lysine side chain residues of tetanus toxoid (TT) were derivatized with TFCS to form the reactive intermediate TT-TFCS through a stable amide bond. The TT-TFCS was covalently condensed to the free imide group to EDC-M-6-H, thus forming through a second stable amide bond the complete structural formulation of the M-TT vaccine.
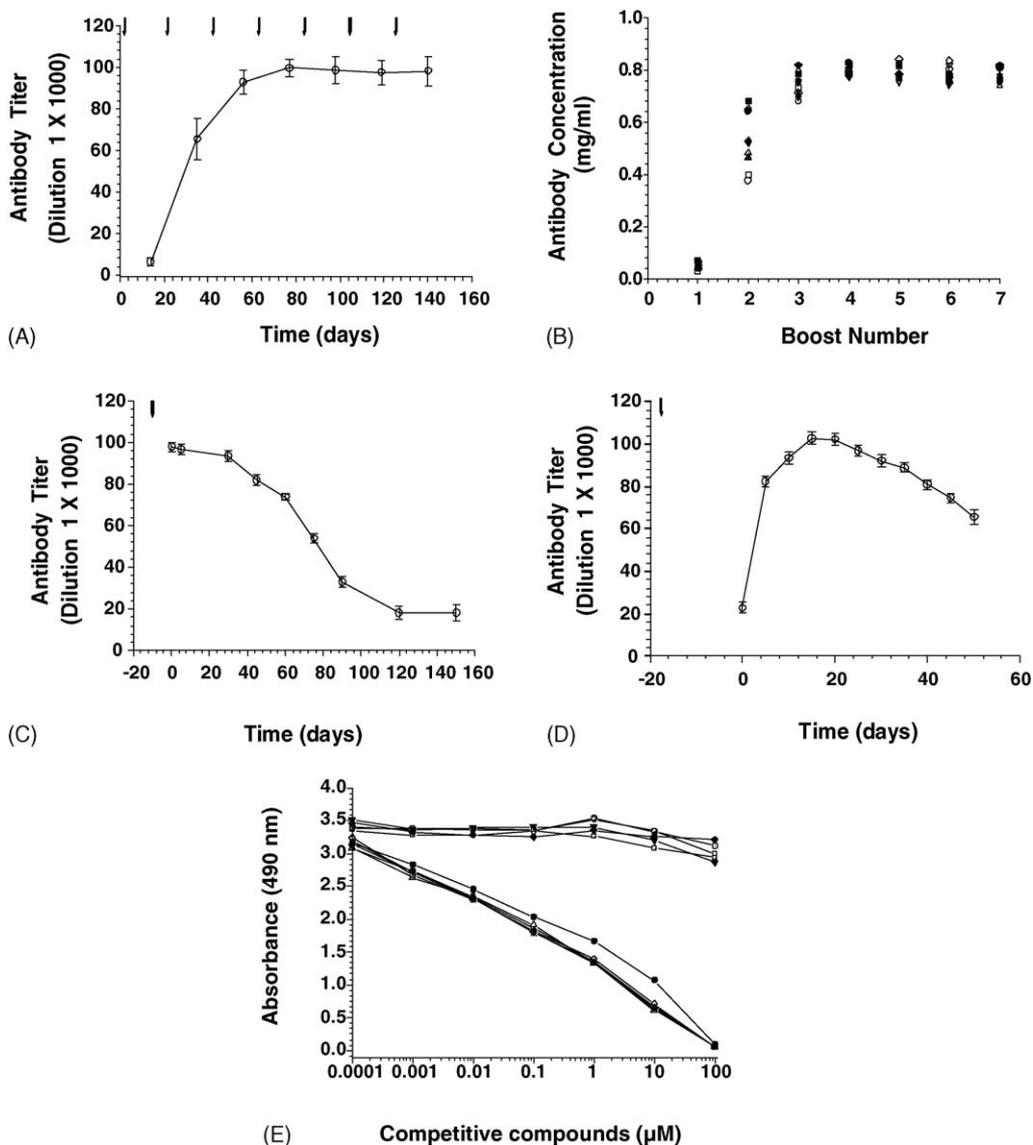
Fig. 2. Characterization of the immune humoral response to M-TT vaccine in actively vaccinated rats. (A) Time-course of the anti-morphine antibody titer response in boosted rats with the M-TT vaccine. The solid vertical arrows point for booster injections and 14 days after antibody titers were measured in the experimental group (empty circles on the graph) (mean ± S.E.M.; $n = 10$). (B) Individual variability of serum antibody levels from same group of animals showed in (A). Symbols (○, □, △, ▽, ◇, ●, ■, ▲, ▼, ◆) are the serum levels (mg/ml) in individual animals. Time-course of the kinetics of decay (C) and recovery (D) of titers of the anti-morphine antibodies. A slow and progressive decay in serum antibody levels (C) was observed after the boost (solid vertical arrow) (mean ± S.E.M.; $n = 10$), reaching detectable levels $\geq 1:20,000$ after 3–4 months and later rapidly recovering to maximal levels ($\approx 1:80–100,000$) within the first 5–14 days after reboost (solid vertical arrow) (D). Titers are expressed on the ordinate axis as the inverse of the sera dilution that gave 50% of the maximal response and points are the mean ± S.E.M. of serum antibody levels from ten vaccinated animals. (E) Analyses of the specificity of anti-morphine antibodies. Anti-morphine antibodies showed equivalent specificities for immunological recognition for heroin (◇), morphine (○), morphine-ovalbumine (△), the neuroactive metabolite, M-6-G (*) and inactive metabolite M-3-G (◊), as well as 6-MAM (●). No significant antibody cross-reactivity was observed for Leu-enkephalin (▼) and β-endorphin (□) including therapeutic opiate compounds such as methadone (◘) naltrexone (□) and buprenorphine (◆). Values of antibody binding to the antigen adsorbed to the solid phase of the ELISAs are expressed as absorbance units at each point of varying concentration of indicated competitors from ten vaccinated animals.

rodent toxicity studies [9–11 and references therein] should be addressed to explore for potential systemic vaccine-induced adverse effects at the level of histophatological damage in organs and blood cells. Animals were immunized with the M-TT conjugate and bled at varying times. The anti-morphine antibodies were monitored by ELISA assays [19]. A progressive increase in the titer of specific

antibodies was observed during the four initial boosts (Fig. 2A). Maximal sustained antibody titers ($\approx 1:100,000$, 0.8 ± 0.2 mg of specific immunoglobulin/ml of sera) were invariably reached after the fourth boost (Fig. 2A and B), and remained sustained over a period of a year with periodic administration of repeated booster. Additionally, a narrow variability of antibody serum levels (geometric

mean ± S.E.M. in mg/ml for boost 1 = 0.052 ± 0.013; boost 2 = 0.53 ± 0.103; boost 3 = 0.75 ± 0.046; boost 4 = 0.81 ± 0.02; boost 5 = 0.80 ± 0.31; boost 6 = 0.78 ± 0.025 and boost 7 = 0.79 ± 0.033) were observed in this group of vaccinated animals after each boost (Fig. 2B). This latter result suggests that individual variation in active antibody production would not impair the M-TT vaccine effectiveness in blunting relapse to opiate consumption (see below in Section 3.4), because based on other animal self-administration studies for cocaine and nicotine, vaccine treatment effectiveness most likely depends upon critical antibody threshold level [9,10]. Furthermore, a noteworthy finding was that the high-titer anti-morphine antibody responses observed in our study was several fold higher than those reported for other vaccines for drugs of abuse tested in rats, and nearly an order of magnitude higher than the best currently used vaccines in humans [9,10]. However, it is worth to mention that such comparisons do not necessarily reflect a superior immunogenic capability of the M-TT vaccine for heroin than other tested addictive vaccine conjugates. For example, use of different vaccine dosing schedules and structurally dissimilar carrier hapten proteins (i.e., tetanus toxoid versus either BSA or B subunit polypeptide from cholera toxin) as well as haptenized drugs (i.e., cocaine and nicotine) may ultimately account for differential immune responses to addictive drugs either in rodent or humans. Furthermore, preliminary studies on synthesis of different morphine conjugates performed by our research group (data not shown) [26,27] were based on the structural model of the morphine-6-hemisuccinyl-BSA conjugate reported by Bonese et al. [12]. In these studies, we used KLH, ovoalbumin, tyroglobulin and BSA as carrier proteins to haptenize morphine through aliphatic spacer linker arms of varying lengths (i.e., 2–6 Å). Overall, a common feature of these latter vaccine models was its low serum drug-specific antibody concentrations elicited (0.02–0.08 mg/ml) when compared to the M-TT vaccine [26,27]. Since morphine by itself is a very poorly immunogenic hapten [19], we could argue that the superior anti-morphine antibody response elicited by our M-TT vaccine could be due to the use of a highly immunogenic carrier protein (tetanus toxoid) [22] and both the nature and length of the spacer linker arm through which morphine is haptenized and acquires enough spatial freedom in the conjugate to become highly antigenic and immunogenic for the immune system.

Thereafter, we characterized the progressive decay of the titers of anti-morphine antibodies in non-boosted immunized animals. Antibody titers showed a significant decay in a time-dependent manner, reaching its lowest levels (≈1:20,000, 0.16 mg/ml) 120 d after re-boost (Fig. 2C). Following the significant antibody titer decay, a rapid recovery of maximal levels (≈1:80,000–1:100,000, 0.6–0.8 mg/ml) was observed within the first 5–14 days during a subsequent re-boost (Fig. 2D). These latter data confirm the effectiveness of the M-TT vaccine to induce immunologic humoral memory to this opiate.

3.3. Characterization of specificity of antibodies

As heroin is a synthetic morphine analogue produced by acetylation of the phenolic 3-hydroxyl and alcoholic 6-hydroxyl groups, blocking morphine through either its 3- or 6-hydroxyl functional groups could be useful for generating antisera unable to distinguish morphine from heroin [28–30]. Thus, the M-TT vaccine (Fig. 1) was synthesized by haptenizing morphine through its 6-hydroxy functional group for generating antibodies with equivalent specificity to heroin and the metabolites from both opiates. Heroin is rapidly metabolized in blood [31] by sequential deacetylation of two separate ester bonds to yield sequentially 6-monoacetylmorphine (6-MAM), morphine and two glucuronide metabolites named morphine-3-βD-glucuronide (M-3-G) and morphine-6-βD-glucuronide (M-6-G) [24]. Interestingly, 6-MAM and M-6-G have intrinsic efficacy to activate mu-type opioid receptors, thereby possibly contributing to the highly addictive properties of heroin [32–35].

A competition ELISA (Fig. 2E) was used to evaluate the specificity of the anti-morphine antibodies. There was no significant recognition for structurally dissimilar therapeutic opiate compounds such as buprenorphine, methadone and naltrexone. An absence of cross-recognition was also observed for a representative opioid peptide sequences such as leu-enkephalin and β-endorphin. Conversely, the antisera recognized with equivalent specificity 6-MAM, M-3-G and M-6-G. These data suggest the immunogenic capability of our M-TT vaccine to generate a humoral response of anti-morphine antibodies displaying equivalent specificities not only for heroin but also for its active metabolites. As antibodies cannot cross the blood-brain barrier, anti-morphine antibodies could represent efficacious pharmacological agents to retain heroin and its active metabolites in the plasma. The absence of cross-recognition of anti-morphine antibodies for endogenous opioid peptides, such as leu-enkephalin and β-endorphin, indicates a potential safe clinical use of the M-TT vaccine, because no cross-reactivity to endogenous opioid peptides would be generated in immunized subjects. Additionally, the lack of antibody cross-recognition to methadone, buprenorphine and naltrexone makes possible to propose a potential combined use of the M-TT vaccine with these agents for treating heroin maintenance [1,3,6].

3.4. Therapeutic effects of the MTT vaccine

Heroin is known to have potent reinforcing effects that contribute to rapid addiction and high rates of relapse after long periods of abstinence [3,6]. Among the many factors that lead to heroin recidivism, re-exposure to the drug itself has been proven to be one of the most powerful in humans and animals [2,4–6]. Therefore, we next explored the efficacy of active immunization with the M-TT vaccine in preventing reacquisition of heroin self-administration in rats. A rat model of relapse was used where animals were trained to self-administer a moderate reinforcing dose of heroin (0.06 mg/kg
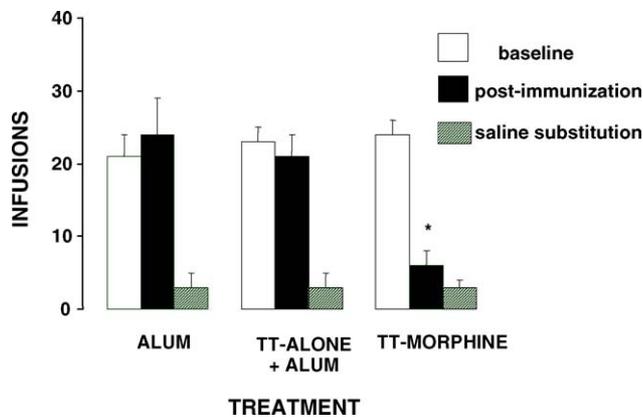
Fig. 3. Active immunization with the M-TT vaccine prevents relapse to heroin taking behavior in rats. After stable baselines of heroin self-administration (0.06 mg/kg per infusion) were acquired, animals were subjected to a period of extinction by substituting saline for the drug, actively vaccinated with the M-TT vaccine ($n = 10$) or control solutions (either alum alone, $n = 10$ or alum plus TT, $n = 10$) and re-exposed to the same unit dose of self-administered heroin. Within each experimental group, a minimum of 10 days of stable baseline response rates at the FR10 in all animals were averaged ($\pm$S.E.M.) and defined as pre-immunization baseline intake of drug (white bars). Pre-immunization saline infusions were averaged ($\pm$S.E.M.) over 5 extinction sessions (stripped bars). Vaccination effects were analyzed by comparing the averaged ($\pm$S.E.M.) heroin infusions during 10–15 post-immunization sessions from M-TT and control vaccinated animals (solid bars). *Significantly different ($F_{2,27} = 15$, $P < 0.005$) from the post-immunization baseline values of control groups immunized with either alum alone (alum) or alum plus carrier protein (TT + alum).

per infusion), subjected to a period of extinction by substituting saline for the drug, vaccinated, and then re-exposed to same unit dose of heroin (Fig. 3). Immunization with the M-TT vaccine induced a high titer response of heroin-specific antibodies in all immunized animals. Serum antibody levels ranged from 0.60 to 0.80 mg/ml 14 days after the last vaccine boost (usually after the fourth). Heroin (0.06 mg/kg) self-administration behavior was fully re-established to pre-immunization levels on the second to third day of drug re-availability, remaining relatively sustained through the 10–15 days of testing at this dose in all control rats. The averaged baseline of infusions/15-days of testing was 23.5 ± 6.5 for animals vaccinated with alum alone and 22.5 ± 4.5 for animals vaccinated with alum plus the carrier protein. In contrast to vaccinated control, animals immunized with the M-TT vaccine did not reacquire heroin-taking behavior throughout the 15 days of testing with same heroin dose. For the group of ten subjects, immunization with the M-TT vaccine significantly reduced ($F_{2,27} = 15$, $P < 0.005$) total infusions (4 ± 2) when compared to vaccinated control. Additionally, there were not significant differences for total infusions after saline substitution in vaccinated control (3 ± 1 for alum and 3 ± 2 for alum plus carrier protein vaccination) and M-TT (3 ± 1) immunized animals. The effectiveness of a vaccine on antibody serum concentration of specific antibodies has been demonstrated for cocaine and nicotine vaccines [9,10], and is likely with a heroin vaccine as well. Thus, a major concern with vaccines for drug addiction in general is that they

can be surmounted by escalating the drug dose. Importantly, rats in this study did not compensate for vaccination with increased self-administration of 0.06 mg/kg heroin. However, the potential surmountability of the antibody-induced blockade with higher doses of self-administered heroin will have to be explored. Central to the potential therapeutic value of the M-TT vaccine is its high immunogenic capacity of inducing a high titer antibody response that does not fluctuate over a wide range and that is at least 10-fold greater that what has been previously described for other drugs of abuse [9,10 and references therein]. A pool of high-titer antisera ($\approx$1:100,000) contained high-affinity antibodies (2.5–$4 \times 10^9\,M^{-1}$) with a combined heroin binding capacity of up to 9–14 μM (0.6–0.8 mg/ml antigen-specific antibody). Such high antibody titers would also predict a low probability of antibody surmountability with increasing unit doses of heroin (i.e., $\approx$0.1–0.8 mg/kg) tested in rodents in other self-administering protocols for this opiate. Additionally, questions such a minimum titer for immunoprotection, ease of saturation of the antibody and the possibility of its recovery from opiate saturation have been deferred in the present work and should be approached in future studies. Collectively, these data would provide valuable immunopharmacological information useful for designing potential future clinical trials.

The M-TT vaccine introduces two novel structural advantages never presented by other morphine conjugates synthesized in the past, including the pioneer M-6-H BSA conjugate used by Bonese et al. [12]. Firstly, the use of a highly immunogenic hapten carrier protein (tetanus toxoid) appropriate and licensed for human vaccination. Secondly, the presence of a longer linker spacer arm of aliphatic residues of carbon atoms through which morphine is covalently linked to hapten carrier protein using two stable amide bonds. Whether the very high serum and sustained antibody concentrations over time attained in our study would be achievable in humans using the M-TT vaccine, who have shown to develop lesser and very variable anti-drug antibody titer response than experimental animals [9–11 and references therein], is an issue that remains to be explored in future Phases I–IIa clinical trials. Although the current studies suggest that the M-TT vaccine represents an attractive candidate medication to test safety and immunogenicity in Phase I clinical trials against heroin and/or morphine, additional detailed studies should be addressed to evaluate potential toxic side effects in other experimental animals (i.e., rabbits or primates). Furthermore, similar to the cocaine vaccine, TA-CD [11,36], a clinical trial program could be developed to further evaluate the safety, immunogenicity, and efficacy of M-TT in human volunteers. Given that the antibodies generated after immunization with M-TT cannot cross the blood-brain barrier, treatment effects are not expected to be psychoactive. Therefore, the M-TT vaccine may serve as a therapeutic adjunct to other pharmacotherapies aimed at heroin addiction treatments to maintain prolonged abstinence to this opiate with classical pharmacological agents [3,6,7]. Moreover, as illicit opiate use is

common among heroin addicts using these treatment modalities [13], having circulating anti-heroin antibodies is likely to reduce the use of such illicit substances, as their reinforcing effects would be blocked. Furthermore, the use of the M-TT vaccine in combination with existing therapies could also be useful to reduce the duration of substituted addiction and development of deleterious side-effects often seen with the long-term use of these pharmacological agents [13] that ultimately contribute to the high incidence of treatment abandon and heroin relapse [13]. For example, naltrexone is not inactive in the absence of exogenous addictive opiates and blocks the action of the body's endogenous opoid peptides (i.e., enkephalins, dynorphins and endorphins), and this can cause negative emotional effects (such as depressed mood), which reduce patient compliance [1–3]. Additionally, clinically documented long-term side effects such as constipation, weight gain, decreased libido and menstrual irregularities (resulting from hyperprolactinemia) have been reported in using methadone for heroin maintenance [37 and references therein]. Finally, the M-TT vaccine could be used as an alternative medication in heroin maintenance treatments in many countries where the medical prescription and authorization of use of pharmacological agents such as methadone and buprenorphine/naloxone are not yet available. Heroin addiction is a complex disease where one of the major problems to be improved is the high incidence of relapse during maintenance to same or other addictive drugs (i.e., cocaine). Thus, in this world of multiple drug abuse/polydrug abuse, it is likely that such complex drug-intake patterns could only be successfully treated by using a combination of both current and novel therapeutic medications (i.e., simultaneous active vaccination against heroin/morphine/cocaine/amphetamines using polyvalent vaccine formulations).

Acknowledgements

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