Induction of Protective and Specific Antibodies against Cocaine by Intranasal Immunisation Using a Glyceride Adjuvant

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The goal of this study was to investigate an intranasal cocaine vaccine containing the mucosal adjuvant macrogol-6-glycerol capylocaprate (RhinoVax). Cocaine–KLH conjugate was prepared and administered in two formulations. Ten mice were immunised intranasally using RhinoVax as adjuvant and ten subcutaneously using aluminium hydroxide as an adjuvant. A negative control group ($n=10$) received unconjugated KLH with RhinoVax intranasally. Specific cocaine antibodies in serum were measured following primary and booster immunisation. Relative antibody responses in serum indicated that the immunisation was successful. Animals were then challenged with cocaine either intranasally or intraperitoneally with subsequent measurement of drug distribution into the serum, brain and olfactory bulb. The cocaine-immunised groups revealed significantly lower cocaine levels in the brain compared to the negative control group. The inhibition of cocaine distribution to the brain in the intranasal immunised group was comparable to that of the subcutaneous immunised group. This was unexpected because the cocaine specific antibody levels in serum were fivefold lower in the intranasal immunised group. However, the presence of mucosal cocaine specific antibodies after intranasal immunisation could play an important role in hindering direct access of cocaine into the brain *via* the olfactory bulb.

Key words cocaine; addiction; intranasal; vaccine; RhinoVax; immunisation

Cocaine is a powerfully addictive psychostimulant drug whose abuse and withdrawal patterns differ from those of other major illicit drugs. There is a widespread cocaine epidemic[1)] largely associated with introduction of "crack" cocaine. While the need to treat such drug dependency is unequivocal, there is no proven pharmacologic therapy to treat cocaine addiction.[2,3)] A number of approaches have been investigated;[2)] one involves active immunisation with an immunogenic cocaine–protein conjugate to promote the production of cocaine specific antibodies.[4—8)] Under normal conditions antibodies cannot cross the blood-brain-barrier. However it is believed that complexation of antibodies with free cocaine may effectively block access of cocaine to the brain.[6,9)]

Cocaine is known to block the dopamine transporters in the central nervous system,[1,10)] with resultant heightened dopaminergic stimulation at critical brain sites. The rapid and extreme rise of dopamine levels in the brain is thought to account for the addictive nature of cocaine.[11)] The euphoric effects of cocaine seem to critically depend on both the total amount of cocaine in the central nervous system and on the rate of entry into the brain.[12,13)] Recent studies indicate that the latter has greater impact on the cocaine dependence.[14)]

Nasal insufflation of cocaine is a popular administration route among drug addicts because the drug is rapidly absorbed from the nasal mucosa.[15)] Cocaine, however, induces intense vasoconstriction at the site of absorption, which may affect the absorption into the systemic circulation.[14)] Following insufflation a substantial amount of the drug is swallowed into the gastrointestinal tract with subsequent absorption and first-pass metabolism.[14)] Cocaine enters the brain from the circulation through the blood-brain-barrier. A paper by Chow *et al.*[15)] also indicates that a small fraction of intranasally administered cocaine can be transported directly from the nasal cavity to the brain via the olfactory system. Therefore, the rapid onset of action observed for insufflated cocaine may be the result of direct uptake into the brain.

The mucosal surfaces of the respiratory and gastrointestinal systems are continuously exposed to exogenous agents[16)] and require effective defense systems to provide necessary protection. Secretory antibodies, together with mucin, play an important role in preventing mucosal invasion by binding exogenous substances.[16—18)] The nasal passage is easily accessible and highly vascularized and therefore constitutes an attractive route for immunisation. The presence of numerous microvilli covering the nasal epithelium generates a large absorption surface. Immune responses can be induced at mucosal sites distant to the site of immunisation, owing to the dissemination of effectors immune cells in the common mucosal immune system.[19)] Intranasal immunisation induces both mucosal and systemic immune responses, which is a potential advantage for cocaine immunisation.[19)] If cocaine specific secretory antibodies are present on the nasal mucosa, they could potentially bind cocaine at its site of administration thereby hindering both systemic absorption and olfactory absorption. Recent animal studies [unpublished] have shown that cocaine intranasal immunisation with RhinoVax as an adjuvant is effective in stimulating local mucosal cocaine specific antibody responses in the buccal mucosa.

Cocaine is a small molecule and must be covalently linked with an immunogenic macromolecule in order to be immunogenic. To ensure Th2 immune responses towards the molecule, the vaccine must be formulated with a suitable adjuvant. The balance between toxicity and immunogenicity of adjuvants is very delicate. Most adjuvants have the inherent problem of being irritating or even toxic (*e.g.* Freund's complete adjuvant and cholera toxin) which limits their use in humans. In 1998 Gizurarson *et al.* published their work on the RhinoVax, mucosal adjuvant system. This adjuvant is nontoxic and has been studied with a number of antigens in various animals as well as in humans.[20,21)] When administered intranasally, the adjuvant was found to stimulate both local and

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systemic immune responses, providing an interesting alternative to parenteral immunisation. The adjuvant consists of a mixture of mono- and diglycerides of caprylic- (C-8) and capricacid (C-10) where all free alcohol groups are pegylated.

The aim of this work was to investigate whether an intranasal cocaine vaccine using RhinoVax as a mucosal adjuvant, could induce specific cocaine antibodies and, if so, whether these antibodies were able to prevent cocaine from entering into the brain.

MATERIALS AND METHODS

Preparation of Cocaine–KLH Conjugate KLH (Imject mariculture keyhole limpet hemocyanin, Pierce Co., Rockford, IL, U.S.A.) was conjugated to cocaine *via* succinic acid spacer arm as follows: Norcocaine was synthesised from cocaine by *N*-demethylation with 1-chloroethylchloroformate as described by Boja *et al*.[22] Cocaine (455 mg; 1.47 mmol) was dissolved in 1,2-dichloroethane (25 ml) under nitrogen atmosphere at room temperature. 1-chloroethyl chloroformate (0.5 ml) was added drop wise and the mixture then refluxed for 24 h. The reaction mixture was evaporated under reduced pressure and the residue dissolved in MeOH and refluxed for 4 h. The solvent was evaporated under reduced pressure and the residue dissolved in CH_2Cl_2 and washed with dilute K_2CO_3 solution. The organic phase was dried with $MgSO_4$, evaporated under reduced pressure and norcocaine was isolated by column chromatography (10% Et_3N in Et_2O). Norcocaine was succinylated by the method described by Swain *et al*.[23] Norcocaine (320 mg; 1.11 mmol), succinic anhydride (220 mg; 2.20 mmol) and triethylamine (0.3 ml) were dissolved in CH_2Cl_2 (8 ml) and refluxed for 18 h. The reaction mixture was washed with dilute aqueous HCl, dried with $MgSO_4$ and the solvents were removed under reduced pressure. The residue was purified with column chromatography (2 : 1 $CHCl_3$–MeOH).

Succinyl cocaine (30 mg) was dispersed in distilled water at 0 °C and dilute NaOH added until all the succinyl cocaine had dissolved. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Pierce Co.) (21.3 mg) was added and five minutes later 20 mg of KLH in 2 ml PBS (pH 7.4) was added. The reaction was left stirring at room temperature overnight. The reaction was dialysed against 3×3 l of PBS (12 h each). Level of haptenation was determined by analysis of the difference in UV absorbance between conjugated and unconjugated KLH.

Animals Thirty 7-week-old female BALB/c mice (M&B A/S, Ry, Denmark) were used. The animals were maintained in a temperature-controlled room (22±2 °C) and kept on a 12-h light–dark cycle. Food and water were available *ad libitum*.

Vaccine Formulation Three preparations were used: (A) Intranasal: 6.4 mg/ml of the Cocaine-KLH conjugate in PBS with 5% v/v RhinoVax (Softigen 767, Sasol GmbH, Witten, Germany); (B) Subcutaneous: 2.2 mg/ml of the Cocaine-KLH conjugate adsorbed to 1.3% aluminum hydroxide (Alhydrogel, Superphos Biosector, Frederiksund, Denmark) in water; this was allowed to reach equilibrium overnight before use; and (C) Negative control: 6.4 mg/ml of unconjugated KLH in water with 5% v/v RhinoVax.

Immunisation Animals were randomly divided into three groups with ten animals in each group. (1) Intranasal cocaine immunisation; 5 μl of preparation (A) was administered into both nostrils. (2) Subcutaneous cocaine immunisation (positive control group); 60 μl of preparation (B) was injected into the anterior dorsal section of the back. (3) Negative control (intranasal KLH immunisation); 5 μl of preparation (C) was administered into both nostrils. The primary immunisation was given at $T=0$ and booster doses at $T=4$ weeks and $T=7$ weeks.

Sampling Blood was drawn from the tail veins of the animals following the primary immunisation ($T=3$ 1/2 week) and again following the booster immunisation ($T=7$ 1/2 week). Approximately 0.1 ml of blood samples were collected (Minicollect®, Greiner labortechnic, Frickenhausen, Germany) and stored at room temperature for 1 h. Then the samples were centrifuged at 3000 rpm for 15 min and serum collected and stored at −20 °C until analysis.

Antibody Measurements ELISA (enzyme-linked immunosorbent assay) was used to measure cocaine specific antibodies in the mouse sera. Succinyl cocaine (0.1 mg/ml; 100 μl in water) was covalently attached to amine activated polystyrene 96 well microtiter plates (Covalink-NH_2, Nunk A/S, Roskilde, Denmark). Freshly prepared solutions of sulfo-NHS (*N*-Hydroxyxulfosuccinimide, Pierce Co.) (3.48 mg/ml in water) and EDC (30.7 mg/ml in water) were subsequently added to each well. The plates were covered and incubated overnight at room temperature with gentle agitation and then washed with water to remove unbound substance.

To avoid unspecific binding, the plates were incubated for 1 h at room temperature with ELISA buffer (0.5% bovine serum albumin and 0.05% Tween 20 in PBS) and then washed with PBS to remove the blocking agent. The samples and standard (benzoyl ecgonine monoclonal antibodies, Biostride Inc., Redwood City, CA, U.S.A.), in several dilutions, were incubated for 1.5 h at room temperature with gentle agitation and then washed with 0.05% Tween 20 in PBS. Horse-radish-peroxidase conjugated rabbit anti-mouse immunoglobulin (Dako P-260, DAKO A/S, Glostrup, Denmark) was added, incubated for 1 h and then washed with 0.05% Tween 20 in PBS. The peroxidase activity was determined with 1,2-*o*-phenylenediamine hydrochloride (DAKO A/S) and the reaction was stopped after 15 min with 0.5 M sulphuric acid. The absorption was measured at 492 nm in a spectrophotometer (Perkin Elmer HTS 7000, U.S.A.).

The concentration of the cocaine specific antibodies was calculated on the basis of a standard curve.

Cocaine Administration and Distribution Measurements The animals were given 1.5 mg/kg cocaine in PBS together with ^{3}H-cocaine (0.02 μCi) (American Radiolabelled Chemicals, St. Louis, MO, U.S.A.) following the booster immunisation ($T=8$ weeks). Five animals in each group received 100 μl volume intraperitoneally and the other five received 10 μl intranasally. After 3 min the mice were sacrificed and blood, brain, and the olfactory bulb were removed. Ultima Gold™ scintillation fluid (10 ml) (Packard, Meriden, CT, U.S.A.) was added to a known amount of serum (0.1—0.3 ml) and stored at room temperature until analysis. Soluene-350™ (0.75 ml) (Packard) was added to each 100 mg of brain tissue and allowed to dissolve at room

temperature for two days. Finally, 1.5 ml of that solution was diluted with Hionic Fluor™ (15 ml) (Packard) and measured for 5 min on a liquid scintillation counter (LKB-Wallac 1214 Rachbeta).

Statistical Analysis The difference between the groups were analyzed by Student's *t*-test. A 95% confidence interval was used and $p<0.05$ was considered significant.

RESULTS

Preparation of Cocaine–KLH Conjugate The conjugation of cocaine to KLH was successful. The level of haptenation was determined by UV-absorbance, which was a very rudimentary assessment. UV-absorption suggested that approximately 200 molecules of cocaine were conjugated to each KLH molecule. This reflects the minimum number since high conjugation of KLH is known to alter the structure of the KLH protein and expose hydrophobic surfaces that can lead to precipitation.[24] The product of the conjugation contained large amounts of precipitate, which is indicative of high conjugation. The precipitate was included in the mixture used for eliciting immune response in the mice.

Antibody Measurements The relative cocaine specific antibody response in serum was measured following both the primary and the booster immunisation, as seen in Table 1. Animals in both cocaine-immunised groups responded fully to the immunisation. Following primary immunization there was a significant increase ($p<0.005$) in cocaine specific antibody level in the subcutaneously immunised group compared to the negative control group (182.5 U/ml relative to 4.5 U/ml). The intranasally immunised group, however, did not show a significant, increase (10.3 U/ml). The booster immunisation resulted in a strong and significant ($p<0.005$) increase in cocaine specific antibodies for both the intranasal and subcutaneous groups. These rose to 179.3 U/ml and 889.1 U/ml respectively.

Distribution Measurements The animals in both cocaine-immunised groups displayed lower levels of drug in the brain following subsequent cocaine administration than the negative control group (Fig. 1). The subcutaneous immunised group showed significant ($p<0.05$) reduction in cocaine distribution to the brain both when cocaine was administered intraperitoneally (Fig. 1A) and intranasally (Fig. 1B), compared to the negative control group. The intranasal immunised group showed significant ($p<0.05$) reduction in cocaine transport into the brain when it was administered intraperitoneally and substantial, but not significant, reduction when administered intranasally. In Fig. 2, the cocaine concentration in the olfactory bulb was compared to the concentration in the remaining brain tissue. In all cases, cocaine levels in the olfactory bulb were higher following intranasal than after intraperitoneal cocaine administration. This difference was insignificant for both cocaine-immunised groups, but the negative control group had significantly ($p<0.005$) higher cocaine concentration in the olfactory bulb after intranasal cocaine administration than after intraperitoneal administration.

DISCUSSION

An intranasal cocaine vaccine using the mucosal adjuvant Rhinovax, has been studied. Animals treated with conjugated-cocaine vaccines responded fully to the cocaine immunisation and demonstrated elevated specific cocaine antibodies in the serum. Upon subsequent administration of cocaine, the previously immunized animals had significantly lower levels of cocaine in the brain compared to the negative control group (*i.e.* unconjugated-cocaine vaccine).

Antibody Response Analysis of specific antibodies against cocaine indicated that the immunisation was success-

Table 1. Total Cocaine Specific Antibody Responses in Serum Following Immunisation of Mice

Immunisation	Primary immunisation $T=3$ 1/2 week (U/ml)	Booster immunisation $T=7$ 1/2 week (U/ml)
Intranasal	10.3±8.2	179.3±113.1
Subcutaneous	182.5±116.2	889.1±557.4
Negative control	4.5±0.2	3.2±2.6

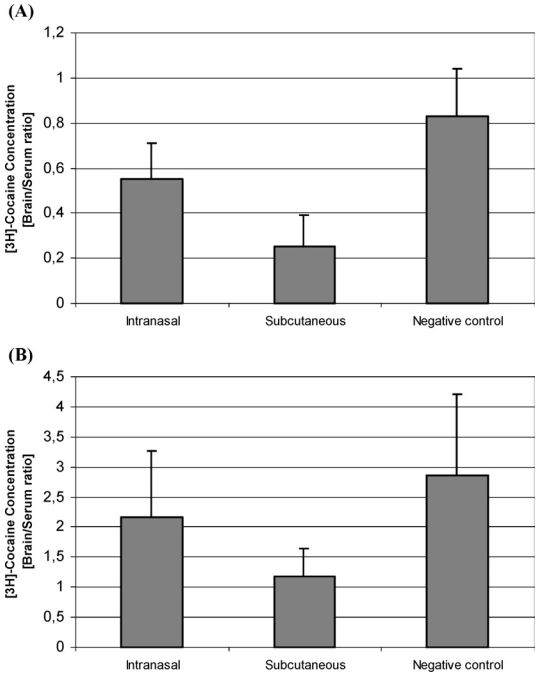

Fig. 1. Cocaine Concentration in the Cerebral Tissue, 3 min after Administration of Cocaine

(A) Intraperitoneal cocaine. (B) Intranasal cocaine.

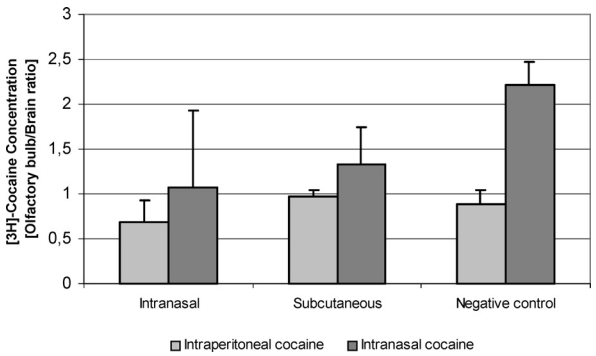

Fig. 2. The Concentration of Cocaine in the Olfactory Bulb, 3 min after the Administration of Cocaine

ful in both conjugated-cocaine immunised groups. There were, however, clear differences in the serum antibody levels depending on the route of immunisation. The intranasal immunisation using RhinoVax as an adjuvant yielded 179 U/ml where the subcutaneous immunisation using aluminium hydroxide yielded 889 U/ml.

The aluminium hydroxide is a typical inducer of pure Th2 immune response,[25] and there are numerous reports in humans and in animals showing its excellence as a primer for vaccinations.[26—28] This is also the case in current study, as the subcutaneous aluminium hydroxide immunisation elicited a strong cocaine specific antibody response in serum following the primary immunisation. The intranasal immunisation was not as efficient in eliciting serum antibodies following the primary immunisation (10 U/ml). One of the functions of the nasal cavity is to tolerate a variety of airborne compounds without inducing immune response to every exogenous substance. Therefore intranasal immunisations are typically not as efficient in eliciting serum antibodies as subcutaneously administered aluminium hydroxide vaccines are.[19,29] By contrast, the intranasal booster administration elevated the antibody responses 18 folds to 179 U/ml, which is equivalent to the levels seen following primary immunisation using the subcutaneous immunisation. The booster effect was less pronounced in the subcutaneous group with only a 5 fold augmentation of the antibody response (from 182.5 U/ml to 889.1 U/ml). Consequently secondary immunization assumes heightened importance for intranasal vaccines since they must overcome the tolerating function of the nasal cavity.

Distribution Study Analysis of the cocaine concentration in the cerebral tissue following subsequent cocaine administration revealed significantly lower concentrations in cocaine-immunised animals, compared to the negative control group.

There were, however, clear differences in the efficacy of the immunisation routes. The subcutaneous immunisation was more powerful in blocking access of cocaine into the brain, irrespective of the delivery route for the cocaine (intranasal or intraperitoneal). Antibodies complexing with the cocaine molecule will block the access of cocaine entering the brain. This is probably attributable to the high levels of cocaine specific antibodies obtained in serum from the subcutaneous group

Although the serum concentration of cocaine specific antibodies in the subcutaneous immunised group was 5 times higher than in the intranasal group, the cocaine levels in the brain of the intranasally immunised animals were only 2 times higher than the subcutaneous group. This indicates that something other than the serum antibodies was hindering cocaine access to the brain in the intranasally immunised group; the most likely explanation being mucosally generated antibodies in the nasal cavity.

There is some evidence suggesting a substantial olfactory absorption of cocaine[15] and if such mechanism is at work then the concentration of cocaine in the olfactory bulb should be higher than in the rest of the brain in the initial pharmacokinetic phase. Cocaine levels in the olfactory bulb 3 min after administration were higher after intranasal than intraperitoneal challenge in all cases, which support this theory. In the negative control group the difference was significant. Concentration of cocaine in the olfactory bulb was found to be 2.21 μg/ml following intranasal administration, compared with 0.88 μg/ml following intraperitoneal administration. However, in the cocaine-immunised groups the difference was not significant. This supports the theory that the antibodies works to block the absorption of cocaine to the brain, both in the circulation and at site of administration. Additional intranasal booster doses, should promote higher concentration of local antibodies on the mucosal surface, thereby effecting stronger blockage at the site of absorption.

The current study showed that intranasally applied cocaine-KLH antigen, together with RhinoVax, was able to produce cocaine specific antibodies, which seemingly blocked the absorption and reduced the level of cocaine entering into the brain. It will be important to study and evaluate the nature of these antibodies, their binding capacity and their function on the mucosal surface as well as inside the body.

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