Fate of Systemically Administered Cocaine in Nonhuman Primates Treated with the dAd5GNE Anticocaine Vaccine

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Abstract

Cocaine use disorders are mediated by the cocaine blockade of the dopamine transporter in the central nervous system (CNS). On the basis of the concept that these effects could be obviated if cocaine were prevented from reaching its cognate receptors in the CNS, we have developed an anticocaine vaccine, dAd5GNE, based on a cocaine analog covalently linked to capsid proteins of an E1[−]E3[−] serotype 5 adenovirus. While the vaccine effectively blocks systemically administered cocaine from reaching the brain by mediating sequestration of the cocaine in the blood, the fact that cocaine also has significant peripheral effects raises concerns that vaccination-mediated redistribution could lead to adverse effects in the visceral organs. The distribution of systemically administered cocaine at a weight-adjusted typical human dose was evaluated along with cocaine metabolites in both dAd5GNE-vaccinated and control nonhuman primates. dAd5GNE sequestration of cocaine to the blood not only prevented cocaine access to the CNS, but also limited access of both the drug and its metabolites to other cocaine-sensitive organs. The levels of cocaine in the blood of vaccinated animals rapidly decreased, suggesting that while the antibody limits access of the drug and its active metabolites to the brain and sensitive organs of the periphery, it does not prolong drug levels in the blood compartment. Gross and histopathology of major organs found no vaccine-mediated untoward effects. These results build on our earlier measures of efficacy and demonstrate that the dAd5GNE vaccine-mediated redistribution of administered cocaine is not likely to impact the vaccine safety profile.

Introduction

COCAINE IS A HIGHLY ADDICTIVE small-molecule drug of abuse with 1.6 million users in the United States (Goldstein *et al.*, 2009; Koob and Volkow, 2010; Substance Abuse and Mental Health Services Administration, 2012). Cocaine inhibits neuronal monoamine transporters, primarily the dopamine reuptake transporter (DAT), leading to accumulation of dopamine in the ventral striatum, caudate, and putamen of the basal ganglia, with consequent increased dopaminergic neurotransmission (Di and Imperato, 1988; Benowitz, 1993; Koob and Volkow, 2010). Repeat use trig-

gers adaptive changes in neuronal circuits underlying reinforcement, reward, and sensitization, associated with addictive behavior (Koob and Volkow, 2010; Kreek *et al.*, 2012).

On the basis of the concept that the effects of cocaine on the central nervous system (CNS) could be obviated if cocaine could be prevented from reaching its cognate receptors in the CNS, we have developed a vaccine (dAd5GNE) consisting of a disrupted serotype 5 E1[−]E3[−] human adenovirus to which the cocaine analog GNE has been covalently linked (Hicks *et al.*, 2011; Wee *et al.*, 2012; Maoz *et al.*, 2013; Cai *et al.*, 2013). Studies in mice, rats, and nonhuman primates have demonstrated that dAd5GNE

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induces high levels of high-affinity anticocaine antibodies that sequester systemically administered cocaine in the blood, preventing cocaine-induced hyperlocomotor activity and sensitization (Hicks *et al.*, 2011; Wee *et al.*, 2012; Maoz *et al.*, 2013).

While the dAd5GNE vaccine prevents access of cocaine to the CNS, cocaine can affect organ systems outside of the CNS. Cocaine disruption of monoamine homeostasis occurs both in the CNS and systemically (Muscholl, 1961; Calligaro and Eldefrawi, 1987; Brody *et al.*, 1990; Lipton *et al.*, 2000; Fowler *et al.*, 2007; Goldstein *et al.*, 2009; Koob and Volkow, 2010). All subtypes of dopamine receptors are expressed in varying proportions in the kidney, adrenal glands, sympathetic ganglia, gastrointestinal tract, blood vessels, and heart (Lackovic and Neff, 1983; Volkow *et al.*, 1992; Benowitz, 1993; Boschetti *et al.*, 2010). Functions mediated by dopamine receptors that are localized outside the CNS include olfaction, vision, and hormone regulation, including adrenocorticotrophic hormone, corticosterone, epinephrine, norepinephrine, prolactin, renin, and aldosterone and regulation of sympathetic tone, renal function, blood pressure, and gastrointestinal motility (Mueller *et al.*, 1990; Volkow *et al.*, 1992; Benowitz, 1993; Gray, 1993; Mendelson *et al.*, 2003). Cocaine also alters the homeostasis of other monoamines in peripheral organs, including the serotonin transporter in the gastrointestinal-digestive tract and heart, and norepinephrine transporters in the adrenal glands, liver, and heart (Nayak *et al.*, 1976; Benowitz, 1993; Lipton *et al.*, 2000; Ding *et al.*, 2003).

In the context that cocaine has widespread systemic effects, one potential adverse consequence of an effective anticocaine vaccine could be the altered distribution of cocaine in the viscera, driven by antibody–cocaine complexes, resulting in high levels of local organ cocaine accumulation and subsequent toxicity (Narvaez *et al.*, 2013). To assess this issue, we evaluated the biodistribution of cocaine and its metabolites in blood and relevant organs and examined all major organs for evidence of toxicology after systemic administration of cocaine to naive and dAd5GNE-vaccinated nonhuman primates.

Methods

dAd5GNE vaccine

The source of the adenovirus capsid proteins for the dAd5GNE vaccine was Ad5 β gal, a recombinant E1a[−], partial E1b[−], and the E3[−] serotype 5 Ad vector with β -galactosidase in the expression cassette (Hicks *et al.*, 2011; Wee *et al.*, 2012). The Ad5 β gal vector was disrupted in 0.5% sodium dodecyl sulfate at 56°C for 45 sec. The cocaine hapten GNE (0.3 mg) was activated overnight at 4°C after the addition of 7.2 μ l charging solution (2.4 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 2 mg of N-hydroxysulfosuccinimide in 4 μ l H₂O and 40 μ l dimethylformamide) (Carrera *et al.*, 1995; Hicks *et al.*, 2011; Wee *et al.*, 2012; Maoz *et al.*, 2013). The conjugation of the disrupted Ad5 β gal vector (200 μ g) with the charged GNE (67 μ g; 300:1 GNE to Ad capsomere molar ratio) was carried out by overnight incubation at 4°C in phosphate-buffered saline (PBS; pH 7.4). The amount of Ad vector proteins was quantified using the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL).

Study design

Six female Rhesus macaques (*Macaca mulatta*) received the vaccine or a control: four were vaccinated with the dAd5GNE vaccine (animals V1, V2, V3, and V4) formulated in 20% Adjuplex™ (Advanced BioAdjuvants LLC, Omaha, NE) and two controls received placebo (animals C1 and C2). The vaccinated animals received dAd5GNE (100 μ g, 0.5 ml) by intramuscular injection to the quadriceps as needed to maintain high anticocaine titers. The vaccine was administered an average 7.2 ± 0.7 times over 12 months and the animals were subjected to cocaine challenges of either 1 mg/kg once every 2 months or on a near-daily basis over a 15-month period. To determine specificity of elicited antibodies for cocaine, venous blood was collected at 8–9 weeks after the initial vaccination and the mean % inhibition by cocaine or cocaine metabolite of anticocaine antibody binding was assayed by competitive ELISA. To assess anticocaine antibody titer at the time of the cocaine biodistribution study, venous blood was collected the same day (V1, V2, V3, C1, and C2) or 1 week before (V4). After collection, the blood was allowed to clot (30 min, 23°C), and centrifuged at $3,000 \times g$ for 15 min. The isolated serum was stored at -20°C . The weight of the animals at the time of necropsy was 6.6 ± 0.5 kg.

Monkeys were anesthetized with ketamine (7.3 ± 0.9 mg/kg) and dexmedetomidine (14 ± 2 μ g/kg). Cocaine (1 mg/kg) was delivered intravenously. Blood samples were collected (0, 2.5, 15, and 60 min) in tubes containing sodium fluoride and potassium oxalate, stored on ice, centrifuged at $3,000 \times g$ for 15 min, and serum was collected. After 60 min, the monkey was euthanized with an intravenous administration of pentobarbital (82 ± 9 mg/kg) and phenytoin (9.8 ± 1.2 mg/kg). The animals were then perfused with 8 liters cold PBS, and the brain and organs were immediately collected. Each organ was harvested separately and evaluated for gross lesions and histopathology by a board-certified veterinary pathologist. Samples of each organ were collected in 0.1–2 g aliquots. Upon collection, serum and organ samples were either immediately flash-frozen in liquid nitrogen, transferred to dry ice for transport, and stored at -80°C or fixed in 10% neutral buffered formalin for histopathology (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/humc). The fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic examination.

Anticocaine antibody titers

To quantify anticocaine titers after vaccination, wells of flat-bottomed 96-well EIA/RIA plates (Corning, New York, NY) were coated with 100 μ l of 1 mg/ml of the cocaine hapten GNE (Hicks *et al.*, 2011; Wee *et al.*, 2012; Cai *et al.*, 2013) conjugated to bovine serum albumin at a molar ratio of 66:1 in bicarbonate buffer, pH 9.4, overnight at 4°C. The plates were washed with 0.05% Tween 20 in PBS (PBS-Tween) and blocked with 5% dry milk in PBS for 30 min at 23°C. Two-fold serial dilutions of serum were added to each well and incubated for 90 min at 23°C. The plates were washed 4 times with PBS-Tween. Diluted horseradish peroxidase-conjugated goat antimonkey IgG (Santa Cruz Biotechnology, Santa Cruz, CA), 100 μ l of 1:2,000 in 1%

dry milk in PBS, was added to each well, and incubated for 90 min at 23°C. The plates were washed four times. Peroxidase substrate (100 μ l/well; Bio-Rad, Hercules, CA) was added and incubated for 15 min at 23°C. The peroxidase reaction was stopped with addition of 2% oxalic acid (100 μ l/well). Absorbance was measured at 415 nm. Anticocaine antibody titers were calculated by interpolation of the log(OD)–log(dilution) with a cutoff value equal to twofold the absorbance of background. To assess dAd5GNE-evoked antibody specificity for cocaine and its metabolites, inhibition of dAd5GNE sera binding to anticocaine ELISA plate was performed in the presence of increasing concentrations (10^{-9} to 10^{-3} M) of cocaine, cocaethylene, benzoylecgonine, norcocaine, ecgonine methyl ester, and procaine.

Cocaine and metabolite analysis

The analysis of cocaine and cocaine metabolites was carried out at the Center for Human Toxicology at the University of Utah. The analysis of the serum and organ

specimens was performed using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS). The basis of the method as developed for cocaine and benzoylecgonine was as described by Lin *et al.* (2001) and methods for assessment of ecgonine methyl ester, and norcocaine as described by Lin *et al.* (2003).

The tissues were first weighed and then homogenized with 9 parts of buffer (0.1 M sodium phosphate pH 6.0 with 1% sodium fluoride). The tissue analysis also included an additional set of positive controls prepared from homogenates of brains from untreated rats (no cocaine administration) that were fortified to 50 ng/ml with cocaine and metabolites. The assay has an analytical range of 2.5–750 ng/ml with an undiluted 1.0 ml aliquot. With dilutions because of homogenization and/or <1.0 ml aliquot size, the lower limits of detection were as follows: serum, 10–25 ng/ml; organs (spleen, lung, liver, and heart), 25 mg/g; and the putamen and adrenal gland, 100 ng/g.

Deuterated cocaine, benzoylecgonine, and ecgonine methyl ester or norcocaine were added to plasma (1.0 ml) as the

A Cocaine and metabolites

B Anti-cocaine antibody specificity

FIG. 1. dAd5GNE generated anticocaine antibody specificity for cocaine compared with cocaine metabolites. (A) Cocaine metabolic pathway (active and nonactive metabolites). (B) dAd5GNE evoked anticocaine antibody specificity for cocaine and active metabolites (cocaethylene and norcocaine) and nonactive metabolites (benzoylecgonine and ecgonine methyl ester). Procaine was used as a low-affinity control. Specificity is displayed as a function of serum anticocaine antibody binding (% inhibition) according to molar concentration of cocaine or cocaine metabolite competitor (M). Each value is % inhibition (average of 3 vaccinated animals $\pm$ SEM).

internal standards. The pH of the plasma was made acidic (pH 4.0) by the addition of acetate buffer and the mixture extracted using mixed-mode octyl and benzoyl sulfonate solid-phase extraction. The eluant was evaporated and reconstituted with methanol/0.1% formic acid in water mixture (1:9) and analyzed by LC-ESI-MS/MS. The mass spectrometer was operated in the selected reaction-monitoring mode. Quadrupole Q1 was set to pass only the MH⁺ ions that are caused to undergo collision-induced dissociation in quadrupole Q2 to abundant product ions as follows: cocaine and cocaine-d³ at m/z 304–182, and 307–185, respectively; benzoylecgonine and benzoylecgonine-d³ at m/z 290–168 and 293–171, respectively; and ecgonine methyl ester and ecgonine methyl ester-d³ at m/z 200–182 and 203–185, respectively. The product ions were selectively filtered by quadrupole Q3 for analysis. The concentration of the analytes was determined from the ratio of the analyte peak area divided by the peak area of the spiked analyte internal standard; the equations from standard curves enabled the conversion to concentration for each cocaine metabolite and cocaine in the human plasma.

The tissues were first weighed and then homogenized with 9 parts of buffer (0.1 M sodium phosphate pH 6.0 with 1% sodium fluoride). From this, a 1 ml aliquot was taken. This resulted in a 1:10 dilution and an analytical range of approximately 10–25 up to 7,500 ng/ml per sample. The tissue analysis also included an additional set of negative controls from the homogenate of the brain from an untreated (no cocaine administration) rat that was established in previous experiments. The lower limits of detection for samples were as follows: serum, 10–25 ng/ml; organs (spleen, lung, liver, and heart), 25 ng/ml; and the putamen and adrenal gland, 100 ng/ml.

Results

Cocaine is metabolized into active, norcocaine, and cocaethylene and inactive, benzoylecgonine, and ecgonine methyl ester that do or do not block the dopamine uptake transporter (Fig. 1A). Vaccination with the dAd5GNE vaccine evoked antibodies with high specificity for cocaine and the active metabolite, cocaethylene (physiological by-product from the covalent interaction of cocaine and alcohol), and with lower specificity for the active metabolite, norcocaine (Fig. 1B) (Dean *et al.*, 1992; Brzezinski *et al.*, 1997; Allen, 2011). The anticocaine antibodies showed lower specificity for the nonactive metabolites, benzoylecgonine and ecgonine methyl ester, and much lower binding affinity for procaine, a low affinity control.

Administration of the dAd5GNE vaccine evoked high titers of anticocaine antibody in the animals ($n=4$), whereas nonvaccinated controls ($n=2$) showed no anticocaine antibody titers (Fig. 2). After intravenous administration of cocaine, examination of the blood compartment revealed that cocaine and metabolites were at the expected levels in control animals, and at higher levels in the blood of vaccinated animals (Fig. 3). Cocaine serum concentration rapidly increased in the first 2.5 min, with vaccinated animals having a threefold or greater increase in cocaine serum levels (mean of $3,354 \pm 456$ ng/ml) compared with controls, demonstrating that the anticocaine antibody retained the cocaine molecule in the blood compartment (Fig. 3A and B). The

FIG. 2. Anticocaine antibody titers of dAd5GNE immunized nonhuman primates (V1, V2, V3, and V4) compared with control (C1 and C2) animals. Displayed is the dAd5GNE-evoked anticocaine antibody titer⁻¹ × 10⁵ in vaccinated (immunized) animals as assessed by ELISA.

serum levels of cocaine of vaccinated animals remained high during the course of the 60 min study and dropped below the level of detection in the controls by 15 min, suggesting that the cocaine was metabolized and/or sequestered into other compartments.

The serum levels of benzoylecgonine and ecgonine methyl ester were similar in vaccinated and control animals at early time points (2.5 and 15 min). At the later time point (60 min), the metabolites (benzoylecgonine, ecgonine methyl ester, and norcocaine) were more abundant in the vaccinated animals, indicating that the cocaine molecule was retained in the blood compartment of vaccinated animals and metabolic breakdown into benzoylecgonine, ecgonine methyl ester, and norcocaine was not slowed (Fig. 3A–H).

Evaluation of cocaine content in the blood, the brain (putamen), and peripheral organs (adrenal gland, spleen, lung, heart, and liver) at 60 min postdrug administration revealed striking differences between controls and vaccinated animals (Fig. 4). At the 60 min time point, both control animals showed no cocaine in the blood compartment but high levels of cocaine in the putamen (Fig. 4A), critical to the addiction pathway and abundant in the high-affinity receptors for cocaine and DAT. Analysis of the adrenal gland confirmed significantly high levels typical for the cocaine-stimulated hypothalamo-pituitary-adrenal axis (Fig. 4A). In control animals, cocaine levels in the heart, kidney, and liver were detectable, with levels in the spleen and lung less consistent but easily detectable (Fig. 4A). In contrast, the vaccinated animals showed high levels of cocaine retained in the blood (Fig. 4B), but there were no detectable levels of cocaine in the putamen or adrenal gland and

FIG. 3. Cocaine and metabolites (ng/ml serum) in the blood over 60 min time course after the administration of cocaine (1 mg/kg, intravenous). (A) Cocaine, controls. (B) Cocaine, immunized. (C) Benzoyllecgonine, control. (D) Benzoyllecgonine, immunized. (E) Ecgonine methyl ester, controls. (F) Ecgonine methyl ester, immunized. (G) Norcocaine, controls. (H) Norcocaine, immunized. Concentrations are displayed on y-ordinate as ng of cocaine or cocaine metabolite per ml of serum at time points indicated (0, 2.5, 15, and 60 min after cocaine administration). Dashed lines show limit of detection (25 ng/ml) of cocaine or metabolite in the serum compartment. Note change in scale on y-ordinate: (A, B) 0–5,000 ng/ml; (C, D) 0–350 ng/ml; (E–H) 0–150 ng/ml.

cocaine levels in the vaccinated animals were lower in the spleen, lung, heart, and kidney relative to control animals. The liver showed similar distribution of cocaine in the vaccinated as compared with the control animals. These findings indicate that vaccination not only blocked cocaine from crossing the blood–brain barrier but also prevented cocaine access to cocaine-sensitive organs in the chest and abdomen (Fig. 4B vs. 4A).

At 60 min after cocaine administration, the major metabolite of cocaine, benzoyllecgonine, was in low abundance in the serum in control animals, with no detectable levels in the putamen, adrenal gland, or spleen (Fig. 5). Benzoyllecgonine was detectable in the lung of one control and detectable levels were also found in the heart, kidney, and liver (Fig. 5A). The vaccinated animals showed abundant levels of benzoyllecgonine in the serum, suggesting that antibody binding to cocaine did not prevent metabolism to the major nonactive metabolite (Fig. 5A and B). Benzoyl-

lecgonine levels were below the limit of detection in the putamen, adrenal gland, and spleen in all vaccinated animals and in liver of three of four vaccinated animals. Only one animal showed minimal levels in the lung and heart (Fig. 5B). These results suggest that vaccination protects not only the brain, but also peripheral organs from cocaine toxicity and also from cocaine metabolite benzoyllecgonine.

The second major metabolite in the breakdown of cocaine, ecgonine methyl ester, was just above the limit of detection in the serum of the two control animals (Fig. 5C) and detectable levels were observed in the brain, as well as all peripheral organs of the two controls. In comparison, in the vaccinated animals, ecgonine methyl ester was below detectable levels in the brain, adrenal gland, and heart (Fig. 5D), but detectable in the spleen and kidney, with minimal levels in the lung and liver of two vaccinated animals.

Cocaine is also demethylated, mostly in the liver, to form the minor active metabolite, norcocaine, and therefore its

FIG. 4. Distribution of cocaine in the blood, brain (putamen), and peripheral organs 1 hr after administration of cocaine (1 mg/kg, intravenous). (A) Control. (B) Immunized. Blood was collected at 60 min, animals were immediately euthanized, and brain and organs were collected. The y-axis shows the drug concentration (ng cocaine/ml serum or g tissue). The x-axis indicates the distribution according to blood compartment (serum), brain (putamen), and peripheral organs (adrenal, spleen, lung, heart, kidney, and liver). Black dashed line shows limit of detection in the putamen and adrenal (100 ng/g). Gray dashed line shows limit of detection (25 ng/ml or ng/g in blood compartment [serum] and specific organs [spleen, lung, heart, kidney, and liver]). The scale on y-ordinate is same for both panels, 0–1,600 ng/ml or ng/g.

detection only in that organ is not unexpected (Fig. 5E). Vaccinated animals showed similar levels of norcocaine in the liver relative to controls (Fig. 5E and F). Higher levels of norcocaine were found in the kidney and to a lesser degree in the lung and spleen of the vaccinated versus control animals, but there were no detectable levels of norcocaine in the brain, heart, or adrenal gland.

Gross and microscopic examination found that one vaccinated animal had tissue necrosis around the site of injection, which may have been caused by nearly concurrent intramuscular injection of sedatives. One vaccinated monkey had a focal area of gliosis in olivary complex in the brain, with inflammatory cell infiltration. The significance of this lesion is unknown. All other findings were deemed typical of Rhesus macaques and none of these findings were considered vaccine related (Supplementary Tables S2 and S3).

In this study, we did not assess the affinity of the antibodies evoked by dAd5GNE for anhydroecgonine methyl ester, a cocaine pyrolysis product of crack cocaine, and thus the distribution of this component in crack cocaine users is unknown.

Discussion

Cocaine is a highly addictive small-molecule drug abused worldwide (Volkow, 2010; Substance Abuse and Mental Health Services Administration, 2012). The allure of cocaine is the rapid euphoria experienced by the user, resulting from rapid access of the molecule to the CNS and blockage of the dopamine transporter resulting in accumulation of dopamine in the synaptic cleft of mesolimbic neurons (Di and Imperato, 1988; Koob and Volkow, 2010). To attenuate the positive subjective effects and the direct reinforcing effects of cocaine, we have developed dAd5GNE, an

anticocaine vaccine designed to prevent administered cocaine from reaching the brain (Hicks *et al.*, 2011; Wee *et al.*, 2012; Maoz *et al.*, 2013). dAd5GNE is comprised of a disrupted serotype 5 adenovirus E1[−]E3[−] gene transfer vector to which GNE (Cai *et al.*, 2013), a cocaine analog, is covalently attached. The vaccine is designed to leverage the strong antiadenovirus immune response in humans to produce high-avidity antibodies against cocaine (Harvey *et al.*, 1999; Chirmule *et al.*, 1999; Hackett *et al.*, 2000; Hicks *et al.*, 2011; Wee *et al.*, 2012; Maoz *et al.*, 2013; Cai *et al.*, 2013). High levels of high-avidity anticocaine antibodies prevent systemically administered cocaine from crossing the blood–brain barrier and entering the CNS and reaching the cognate receptors in the brain (Maoz *et al.*, 2013; Cai *et al.*, 2013). With the goal of assessing the fate of systemically administered cocaine in the context of vaccination with dAd5GNE, we have evaluated the levels of cocaine and its metabolites in the brain, blood, and peripheral organs after intravenous administration of cocaine to nonhuman primates vaccinated with dAd5GNE after chronic exposure to cocaine. The results demonstrate that dAd5GNE vaccination significantly shielded both the CNS and peripheral organs, including the adrenal gland, spleen, lung, kidney, and liver from cocaine and the metabolites benzoylecgonine, ecgonine methyl ester. No findings in peripheral organs suggested vaccine-mediated toxicity.

Cocaine metabolism

To be effective, the anticocaine antibodies evoked by the dAd5GNE vaccine must block not only cocaine itself but also its active cocaine metabolites from access to the CNS. Cocaine is extensively metabolized, with less than 10% excreted in the urine (Barnett *et al.*, 1981; Jeffcoat *et al.*, 1989;

FIG. 5. Distribution of cocaine metabolites (benzoylecgonine, ecgonine methyl ester, and norcocaine) in the blood, brain (putamen), and peripheral organs (adrenal, spleen, lung, heart, kidney, and liver) 1 hr after administration of cocaine (1 mg/kg, intravenous). (A) Benzoylecgonine, control. (B) Benzoylecgonine, immunized. (C) Ecgonine methyl ester, controls. (D) Ecgonine methyl ester, immunized. (E) Norcocaine, controls. (F) Norcocaine, immunized. Blood was collected at 60 min, animals were immediately euthanized, and brain and organs were collected. The y-axis shows the concentration of drug metabolite (ng of cocaine metabolite per ml of serum or g of tissue). The x-axis indicates the distribution according to blood compartment (serum), brain (putamen), and peripheral organs (adrenal, spleen, lung, heart, kidney, and liver). Black dashed line shows limit of detection in the putamen and adrenal (100 ng/g). Gray dashed line shows limit of detection (25 ng/ml or ng/g) in blood compartment (serum) and specific organs (spleen, lung, heart, kidney, and liver). Note that scale on y-ordinate is same for all panels, 0–350 ng/ml or ng/g.

Benowitz, 1993; Scheidweiler *et al.*, 2010; Bystrowska *et al.*, 2012). Enzymatic and nonenzymatic hydrolysis to benzoylecgonine accounts for an average of 45% of cocaine, while enzymatic hydrolysis to ecgonine methyl ester accounts for about 40% of cocaine metabolism, and microsomal oxidative metabolism of cocaine to norcocaine only accounts for a small percentage (1–3%) of the metabolized drug (Jeffcoat *et al.*, 1989; Benowitz, 1993; Gorelick, 1997; Scheidweiler *et al.*, 2010; Yao *et al.*, 2013). These cocaine metabolites and cocaine *per se* are potentially toxic to not only the CNS but also the heart, liver, and other organs (Kloss *et al.*, 1983; Silva *et al.*, 1991; Benowitz, 1993; Hollander, 1995; Restrepo *et al.*, 2007). Therefore, vaccine-mediated redistribution of administered cocaine and/or its metabolites represents a potential safety concern.

Distribution of cocaine and cocaine metabolites in the context of dAd5GNE immunization

Cocaine rapidly distributes to body tissues with a moderate affinity (Jeffcoat *et al.*, 1989; Benveniste *et al.*, 2005). The heart and cardiovascular systems are particularly sensitive to cocaine accumulation (Benowitz, 1993; Hollander, 1995; Connors and Hoffman, 2013; Pilgrim *et al.*, 2013; Ibrahim *et al.*, 2013). In the heart, cocaine inhibits membrane permeability to sodium during depolarization of cell membranes, thereby blocking the transmission of fast sodium currents and the electrical impulse of sensory neurons. This sedative effect on heart tissue can contribute to the arrhythmic potential of cocaine. It also activates α -adrenoreceptors in the heart causing vasoconstriction in

coronary arteries, increases the concentration of calcium in cardiac myocytes, and, at high dose, inhibits norepinephrine reuptake that may affect cardiac action potentials (Muscholl, 1961; Derlet and Albertson, 1989; Brody *et al.*, 1990; Rump *et al.*, 1995; Lipton *et al.*, 2000). Autonomic and neuromuscular effects include tachycardia, hypertension, hyperthermia, respiratory arrest, and cardiovascular collapse (Derlet and Albertson, 1989; Lipton *et al.*, 2000; Lange and Hillis, 2001). In addition to its profound effects on the brain and the heart, cocaine also has significant effects on peripheral organs, altering metabolism in the blood vessels, adrenal glands, and kidneys within minutes of cocaine administration (Benowitz, 1993; Blake *et al.*, 1994; Mendelson *et al.*, 2003). In these organs, butylcholinesterase and hydrolases enzymatically degrade the cocaine molecule (Barnett *et al.*, 1981; Jeffcoat *et al.*, 1989; Dean *et al.*, 1992; Brzezinski *et al.*, 1997; Gorelick, 1997), and these cocaine degradation products are potentially toxic in the organs in which they accumulate (Kloss *et al.*, 1983; Silva *et al.*, 1991; Lipton *et al.*, 2000).

The data in the present study demonstrate that dAd5GNE-evoked antibodies prevent cocaine from reaching the brain and other critical peripheral organs without delaying metabolic breakdown of the cocaine. In the serum of vaccinated animals, the levels of cocaine were reduced by threefold in 1 hr, where it is degraded into its major metabolite, benzoylecgonine, and to a lesser degree ecgonine methyl ester and norcocaine. At the same time, in vaccinated animals, cocaine did not accumulate in the brain or peripheral organs. dAd5GNE vaccination also reduced the accumulation of cocaine and its metabolites in peripheral tissues. The heart is not the only organ sensitive to cocaine-induced α -adrenoreceptor activation and the release of catecholamines, norepinephrine, and epinephrine (Virmani *et al.*, 1988; Derlet and Albertson, 1989; Brody *et al.*, 1990; Lipton *et al.*, 2000). Cocaine-associated ischemic injury has also been documented in many peripheral organs, including the muscle, kidney, placenta, large intestine, and liver (Muscholl, 1961; Benowitz, 1993; Gray, 1993; Lange and Hillis, 2001; Restrepo *et al.*, 2007). dAd5GNE immunization reduced cocaine, benzoylecgonine, and ecgonine methyl ester distribution in the brain, adrenal glands, spleen, lung, heart, and kidney. The liver of dAd5GNE-vaccinated animals did not show increased levels of cocaine and its metabolite, norcocaine, and lower levels of benzoylecgonine and ecgonine methyl ester were observed. It is likely, therefore, that dAd5GNE would protect the brain, heart, lungs, and spleen from cocaine-induced vasoconstriction and not result in adverse effects on the liver or kidney.

In previous studies, we have shown that dAd5GNE vaccination results in high-titer, high-affinity anticocaine antibodies that can abrogate cocaine-mediated locomotor behaviors in mice and rats, alter drug self-administration in rats, and bind and sequester cocaine in the blood compartment in nonhuman primates such that cocaine binding in the CNS remains below the threshold of reinforcement (Volkow *et al.*, 1997; Wee *et al.*, 2012; Maoz *et al.*, 2013; De *et al.*, 2013). In the present study, we demonstrate that the vaccine protects not only the brain but also the relevant peripheral organs from cocaine and its active metabolites without toxicity. If these findings are replicated in human clinical

trials, dAd5GNE may prove to be a therapeutic option for cocaine addiction.

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Author Disclosure Statement

No conflict of interest declared by any of the authors.

References

- Allen, K.R. (2011). Screening for drugs of abuse: which matrix, oral fluid or urine? *Ann. Clin. Biochem.* 48, 531–541.
- Barnett, G., Hawks, R., and Resnick, R. (1981). Cocaine pharmacokinetics in humans. *J. Ethnopharmacol.* 3, 353–366.
- Benowitz, N.L. (1993). Clinical pharmacology and toxicology of cocaine. *Pharmacol. Toxicol.* 72, 3–12.
- Benveniste, H., Fowler, J.S., Rooney, W., *et al.* (2005). Maternal and fetal 11C-cocaine uptake and kinetics measured *in vivo* by combined PET and MRI in pregnant nonhuman primates. *J. Nucl. Med.* 46, 312–320.
- Blake, M.J., Buckley, A.R., Buckley, D.J., *et al.* (1994). Neural and endocrine mechanisms of cocaine-induced 70-kDa heat shock protein expression in aorta and adrenal gland. *J. Pharmacol. Exp. Ther.* 268, 522–529.
- Boschetti, M., Gatto, F., Arvigo, M., *et al.* (2010). Role of dopamine receptors in normal and tumoral pituitary corticotrophic cells and adrenal cells. *Neuroendocrinology* 92 Suppl 1, 17–22.
- Brody, S.L., Slovis, C.M., and Wrenn, K.D. (1990). Cocaine-related medical problems: consecutive series of 233 patients. *Am. J. Med.* 88, 325–331.
- Brzezinski, M.R., Spink, B.J., Dean, R.A., *et al.* (1997). Human liver carboxylesterase hCE-1: binding specificity for cocaine, heroin, and their metabolites and analogs. *Drug Metab. Dispos.* 25, 1089–1096.
- Bystrowska, B., Adamczyk, P., Moniczewski, A., *et al.* (2012). LC/MS/MS evaluation of cocaine and its metabolites in different brain areas, peripheral organs and plasma in cocaine self-administering rats. *Pharmacol. Rep.* 64, 1337–1349.
- Cai, X., Whitfield, T., Hixon, M.S., *et al.* (2013). Probing active cocaine vaccination performance through catalytic and non-catalytic hapten design. *J. Med. Chem.* 56, 3701–3709.
- Calligaro, D.O., and Eldefrawi, M.E. (1987). Central and peripheral cocaine receptors. *J. Pharmacol. Exp. Ther.* 243, 61–68.
- Carrera, M.R., Ashley, J.A., Parsons, L.H., *et al.* (1995). Suppression of psychoactive effects of cocaine by active immunization. *Nature* 378, 727–730.
- Chirmule, N., Propert, K., Magosin, S., *et al.* (1999). Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther.* 6, 1574–1583.

- Connors, N.J., and Hoffman, R.S. (2013). Experimental treatments for cocaine toxicity: a difficult transition to the bedside. *J. Pharmacol. Exp. Ther.* 347, 251–257.
- De, B.P., Pagovich, O.E., Hicks, M.J., *et al.* (2013). Disrupted adenovirus-based vaccines against small addictive molecules circumvent anti-adenovirus immunity. *Hum. Gene Ther.* 24, 58–66.
- Dean, R.A., Harper, E.T., Dumaual, N., *et al.* (1992). Effects of ethanol on cocaine metabolism: formation of cocaethylene and norcocaethylene. *Toxicol. Appl. Pharmacol.* 117, 1–8.
- Derlet, R.W., and Albertson, T.E. (1989). Emergency department presentation of cocaine intoxication. *Ann. Emerg. Med.* 18, 182–186.
- Di, C.G., and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. USA* 85, 5274–5278.
- Ding, Y.S., Lin, K.S., Garza, V., *et al.* (2003). Evaluation of a new norepinephrine transporter PET ligand in baboons, both in brain and peripheral organs. *Synapse* 50, 345–352.
- Fowler, J.S., Kroll, C., Ferrieri, R., *et al.* (2007). PET studies of d-methamphetamine pharmacokinetics in primates: comparison with l-methamphetamine and (-)-cocaine. *J. Nucl. Med.* 48, 1724–1732.
- Goldstein, R.A., DesLauriers, C., Burda, A., *et al.* (2009). Cocaine: history, social implications, and toxicity: a review. *Semin. Diagn. Pathol.* 26, 10–17.
- Gorelick, D.A. (1997). Enhancing cocaine metabolism with butyrylcholinesterase as a treatment strategy. *Drug Alcohol Depend.* 48, 159–165.
- Gray, J.D. (1993). Medical consequences of cocaine. *Can. Fam. Physician* 39, 1975–1981.
- Hackett, N.R., Kaminsky, S.M., Sondhi, D., *et al.* (2000). Antivector and antitransgene host responses in gene therapy. *Curr. Opin. Mol. Ther.* 2, 376–382.
- Harvey, B.G., Hackett, N.R., El-Sawy, T., *et al.* (1999). Variability of human systemic humoral immune responses to adenovirus gene transfer vectors administered to different organs. *J. Virol.* 73, 6729–6742.
- Hicks, M.J., De, B.P., Rosenberg, J.B., *et al.* (2011). Cocaine analog coupled to disrupted adenovirus: a vaccine strategy to evoke high-titer immunity against addictive drugs. *Mol. Ther.* 19, 612–619.
- Hollander, J.E. (1995). The management of cocaine-associated myocardial ischemia. *N. Engl. J. Med.* 333, 1267–1272.
- Ibrahim, M., Maselli, D.J., Hasan, R., *et al.* (2013). Safety of beta-blockers in the acute management of cocaine-associated chest pain. *Am. J. Emerg. Med.* 31, 613–616.
- Jeffcoat, A.R., Perez-Reyes, M., Hill, J.M., *et al.* (1989). Cocaine disposition in humans after intravenous injection, nasal insufflation (snorting), or smoking. *Drug Metab. Dispos.* 17, 153–159.
- Kloss, M.W., Rosen, G.M., and Rauckman, E.J. (1983). N-demethylation of cocaine to norcocaine. Evidence for participation by cytochrome P-450 and FAD-containing monooxygenase. *Mol. Pharmacol.* 23, 482–485.
- Koob, G.F., and Volkow, N.D. (2010). Neurocircuitry of addiction. *Neuropsychopharmacology* 35, 217–238.
- Kreek, M.J., Levran, O., Reed, B., *et al.* (2012). Opiate addiction and cocaine addiction: underlying molecular neurobiology and genetics. *J. Clin. Invest.* 122, 3387–3393.
- Lackovic, Z., and Neff, N.H. (1983). Minireview. Evidence that dopamine is a neurotransmitter in peripheral tissues. *Life Sci.* 32, 1665–1674.
- Lange, R.A., and Hillis, L.D. (2001). Cardiovascular complications of cocaine use. *N. Engl. J. Med.* 345, 351–358.
- Lin, S.N., Moody, D.E., Bigelow, G.E., *et al.* (2001). A validated liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry method for quantitation of cocaine and benzoylecgonine in human plasma. *J. Anal. Toxicol.* 25, 497–503.
- Lin, S.N., Walsh, S.L., Moody, D.E., *et al.* (2003). Detection and time course of cocaine N-oxide and other cocaine metabolites in human plasma by liquid chromatography/tandem mass spectrometry. *Anal. Chem.* 75, 4335–4340.
- Lipton, J.W., Mangan, K.P., and Silvestri, J.M. (2000). Acute cocaine toxicity: pharmacology and clinical presentations in adult and pediatric populations. *J. Pharm. Practice* 13, 159–169.
- Maoz, A., Hicks, M.J., Vallabhjousula, S., *et al.* (2013). Adenovirus capsid-based anti-cocaine vaccine prevents cocaine from binding to the nonhuman primate CNS dopamine transporter. *Neuropsychopharmacology* 38, 2170–2178.
- Mendelson, J.H., Sholar, M.B., Mutschler, N.H., *et al.* (2003). Effects of intravenous cocaine and cigarette smoking on luteinizing hormone, testosterone, and prolactin in men. *J. Pharmacol. Exp. Ther.* 307, 339–348.
- Mueller, P.D., Benowitz, N.L., and Olson, K.R. (1990). Cocaine. *Emerg. Med. Clin. North Am.* 8, 481–493.
- Muscholl, E. (1961). Effect of cocaine and related drugs on the uptake of noradrenaline by heart and spleen. *Br. J. Pharmacol. Chemother.* 16, 352–359.
- Narvaez, J.C., Magalhaes, P.V., Fries, G.R., *et al.* (2013). Peripheral toxicity in crack cocaine use disorders. *Neurosci. Lett.* 544, 80–84.
- Nayak, P.K., Misra, A.L., and Mule, S.J. (1976). Physiological disposition and biotransformation of (3H) cocaine in acutely and chronically treated rats. *J. Pharmacol. Exp. Ther.* 196, 556–569.
- Pilgrim, J.L., Woodford, N., and Drummer, O.H. (2013). Cocaine in sudden and unexpected death: a review of 49 post-mortem cases. *Forensic Sci. Int.* 227, 52–59.
- Restrepo, C.S., Carrillo, J.A., Martinez, S., *et al.* (2007). Pulmonary complications from cocaine and cocaine-based substances: imaging manifestations. *Radiographics* 27, 941–956.
- Rump, A.F., Theisohn, M., and Klaus, W. (1995). The pathophysiology of cocaine cardiotoxicity. *Forensic Sci. Int.* 71, 103–115.
- Scheidweiler, K.B., Spargo, E.A., Kelly, T.L., *et al.* (2010). Pharmacokinetics of cocaine and metabolites in human oral fluid and correlation with plasma concentrations after controlled administration. *Ther. Drug Monit.* 32, 628–637.
- Silva, M.O., Roth, D., Reddy, K.R., *et al.* (1991). Hepatic dysfunction accompanying acute cocaine intoxication. *J. Hepatol.* 12, 312–315.
- Substance Abuse and Mental Health Services Administration. (2012). *Results from the 2011 National Survey on Drug Use and Health: Mental Health Findings*. NSDUH Series H-45, HHS Publication NO. (SMA) 12-4725, Rockville, MD.
- Virmani, R., Robinowitz, M., Smialek, J.E., *et al.* (1988). Cardiovascular effects of cocaine: an autopsy study of 40 patients. *Am. Heart J.* 115, 1068–1076.
- Volkow, N.D. (2010). *Cocaine: Abuse and Addiction*. NIH Publication 10-4166, 1-8. National Institutes of Health National Institute on Drug Abuse, Bethesda, MD.

- Volkow, N.D., Fowler, J.S., Wolf, A.P., *et al.* (1992). Distribution and kinetics of carbon-11-cocaine in the human body measured with PET. *J. Nucl. Med* 33, 521–525.
- Volkow, N.D., Wang, G.J., Fischman, M.W., *et al.* (1997). Relationship between subjective effects of cocaine and dopamine transporter occupancy. *Nature* 386, 827–830.
- Wee, S., Hicks, M.J., De, B.P., *et al.* (2012). Novel cocaine vaccine linked to a disrupted adenovirus gene transfer vector blocks cocaine psychostimulant and reinforcing effects. *Neuropsychopharmacology* 37, 1083–1091.
- Yao, D., Shi, X., Wang, L., *et al.* (2013). Characterization of differential cocaine metabolism in mouse and rat through metabolomics-guided metabolite profiling. *Drug Metab. Dispos.* 41, 79–88.

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