

Editor's Summary

Extinguishing Addiction

Those who smoke and try to quit have variously described their feelings as a never-ending hunger, terror, immense panic, rage, and the loss of a loved one —reactions that stem from nicotine addiction and withdrawal. Given cigarette smoke's destructive effects on health and cost to society, a means of overcoming such addiction would be enormously beneficial. For most smokers, current antismoking therapies fail to work. One newer idea is an anti-nicotine vaccine; in this approach, nicotine (coupled to a larger molecule) is administered, generating an immune response. The resulting anti-nicotine antibodies bind the nicotine from cigarette smoke in the blood, intercepting the drug before it affects reward centers in the brain. In disappointing clinical trials, however, such vaccines result in variable antibody titers and have only limited success in halting smoking, with best results seen in people with the strongest antibody response. Hicks *et al.* now describe a different tactic to get to the same end; they use gene transfer to attain persistent, high levels of anti-nicotine antibodies in smokers' blood.

The researchers constructed an adeno-associated virus–based vector that expressed a high-affinity anti-nicotine monoclonal antibody. Mice injected with a single dose of this vector made high antibody titers (which were in 40-fold molar excess over the nicotine concentrations seen in people who smoke continuously), which remained high for at least 18 weeks. When these mice were injected with nicotine, the antibodies effectively sequestered this compound in the blood: nicotine concentrations in the brain were only 15% of those in brains of mice that did not express the antibodies. Furthermore, the usual nicotine-induced changes in blood pressure, heart rate, and locomotor activity were abolished or greatly reduced in mice that expressed the anti-nicotine antibodies.

Further work will be needed to test this vector in a rodent model trained to self-administer nicotine, because the mice in this study were not addicted to the drug. Successful results from such a test would then support investigating this approach in clinical trials.

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ADDICTION

AAV-Directed Persistent Expression of a Gene Encoding Anti-Nicotine Antibody for Smoking Cessation

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Current strategies to help tobacco smokers quit have limited success as a result of the addictive properties of the nicotine in cigarette smoke. We hypothesized that a single administration of an adeno-associated virus (AAV) gene transfer vector expressing high levels of an anti-nicotine antibody would persistently prevent nicotine from reaching its receptors in the brain. To test this hypothesis, we constructed an AAVrh.10 vector that expressed a full-length, high-affinity, anti-nicotine antibody derived from the Fab fragment of the anti-nicotine monoclonal antibody NIC9D9 (AAVantiNic). In mice treated with this vector, blood concentrations of the anti-nicotine antibody were dose-dependent, and the antibody showed high specificity and affinity for nicotine. The antibody shielded the brain from systemically administered nicotine, reducing brain nicotine concentrations to 15% of those in naïve mice. The amount of nicotine sequestered in the serum of vector-treated mice was more than seven times greater than that in untreated mice, with 83% of serum nicotine bound to immunoglobulin G. Treatment with the AAVantiNic vector blocked nicotine-mediated alterations in arterial blood pressure, heart rate, and locomotor activity. In summary, a single administration of a gene transfer vector expressing a high-affinity anti-nicotine monoclonal antibody elicited persistent (18 weeks), high titers of an anti-nicotine antibody that obviated the physiologic effects of nicotine. If this degree of efficacy translates to humans, AAVantiNic could be an effective preventative therapy for nicotine addiction.

INTRODUCTION

Cigarette smoking is a common addiction, with significant societal effects. About 20% of the adults in the United States smoke cigarettes, and cigarette smoking accounts for one of every five deaths in the United States (1). Cigarette smoke causes chronic obstructive pulmonary disease and lung cancer, and smoking is associated with an increased risk of cardiovascular disease and a variety of nonlung neoplasms (2–5). Smoking-related health care and loss of productivity costs in excess of \$193 billion annually in the United States (5).

Although each puff of cigarette smoke contains more than 4000 chemicals, the addictive properties of cigarette smoking derive from nicotine, a 162-dalton alkaloid that represents 0.6 to 3.0% of the dry weight of tobacco (6–8). Most nicotine is pyrolyzed at the cigarette tip, but each cigarette typically delivers to the smoker 1.0 to 1.5 mg of nicotine, which passes across the alveoli and into the blood stream, taking about 10 to 19 s to reach the brain (9–11). There, nicotine binds to the nicotinic acetylcholine receptor, triggering the conversion of L-tyrosine to dopamine, with resulting pleasure, reduced stress, alterations in blood pressure and heart rate, heightened alertness, and increased ability to process information (12–14).

Despite the devastating health effects of nicotine addiction, current strategies of drug intervention and counseling to help smokers quit are

mostly ineffective, with a 70 to 80% recidivism rate within 6 months (15). Current antismoking medications include nicotine replacement therapies, varenicline (a nicotinic receptor partial agonist), and bupropion (an antidepressant) (16–18), but none have demonstrated high rates of efficacy and some have the potential for serious side effects; for example, varenicline has recently been associated with adverse cardiovascular effects (15, 17, 19). One approach to treating nicotine addiction has been to develop an anti-nicotine vaccine, in which anti-nicotine antibodies bind to nicotine in the blood, preventing the drug from crossing the blood-brain barrier and reaching its cognate receptors in the brain (20, 21). Vaccines have had limited success, possibly as a result of failure to evoke a sufficiently high titer of a high-affinity antibody to nicotine. We therefore hypothesized that an adeno-associated viral gene transfer vector could be designed to express a known, high-affinity anti-nicotine antibody at titers that would prevent nicotine from reaching the brain. Because adeno-associated virus (AAV) vectors can mediate persistent expression, we expected that this approach would require only a single vaccine administration. To evaluate this strategy, we generated AAVrh.10antiNic.Mab (referred to as AAVantiNic), a serotype rh.10 AAV expressing NIC9D9, a high-affinity anti-nicotine monoclonal antibody (22, 23).

RESULTS

Synthesis and characterization of AAVantiNic

Human embryonic kidney (HEK) 293 cells infected with the AAVantiNic vector (Fig. 1A) secreted immunoglobulin G (IgG) antibody, as demonstrated by Coomassie blue-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis (Fig. 1, B and C).

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To assess the ability of AAVantiNic to express and maintain high titers of anti-nicotine antibody in serum, we injected C57BL/6 mice intravenously with AAVantiNic at three doses: 10^9 , 10^{10} , or 10^{11} genome copies (gc). Using an anti-nicotine enzyme-linked immunosorbent assay (ELISA), we demonstrated the dose dependence of the antibody, with the 10^{11} gc group showing the highest serum concentrations of antibody at a mean titer of 1.1 ± 0.2 mg/ml at week 9 (Fig. 2A). This same dose generated a high antibody titer at 4 weeks (0.9 ± 0.1 mg/ml), which remained high until 18 weeks (1.3 ± 0.1 mg/ml), the longest time point evaluated (Fig. 2B). A competitive ELISA showed that the expressed anti-nicotine antibody had a higher affinity for nicotine than for the nicotine metabolites nornicotine and cotinine (Fig. 2C). The K_d (dissociation constant) for nicotine was 43 ± 20 nM (Fig. 2D).

Retention of nicotine to the blood compartment in AAVantiNic-treated mice

To test whether AAVantiNic could express sufficient anti-nicotine antibody to shield the brain from systemically administered nicotine, we challenged AAVantiNic-treated mice intravenously with [3 H]nicotine (11, 24) (Fig. 3). One minute after administration, total serum nicotine concentrations in AAVantiNic-treated mice (516 ng/ml) were 7.2 times higher than in naïve control mice (71.7 ng/ml) ($P < 10^{-5}$). In the AAVantiNic-treated mice, 83% of the serum nicotine was bound to IgG. Conversely, nicotine concentrations in the brain of AAVantiNic-treated mice (12.2 ng/g) were 15% of those in nontreated naïve control mice (79.2 ng/g), and the ratio of nicotine in the blood to that of the

brain was 47 times greater in the AAVantiNic-vaccinated mice than in the controls ($P < 10^{-5}$).

Suppression of the cardiovascular effects of nicotine in AAVantiNic-treated mice

Systemic administration of nicotine robustly changed cardiovascular parameters in mice treated with the AAV control vector. Nicotine caused a 37% reduction in mean arterial pressure and a 46% reduction in heart rate in the control mice within 25 min (Fig. 4). In contrast, nicotine did not induce these blood pressure and heart rate responses in mice treated with AAVantiNic. As a control, we administered phosphate-buffered saline (PBS) in place of nicotine to AAVantiNic-treated mice; no changes were seen in the mean arterial pressure or heart rate.

Suppression of nicotine-induced locomotor behavior in AAVantiNic-treated mice

To determine whether AAVantiNic prevents nicotine-induced suppression of locomotion, we repeatedly challenged AAVantiNic-treated mice (4 to 7 weeks after vector administration) subcutaneously with 12.5 μ g (0.5 mg/kg) of nicotine. Ambulatory activity was assessed for 15 min after nicotine administration for each of 10 challenges for 3 weeks (Fig. 5A). Naïve control mice injected with nicotine showed a marked nicotine-dependent decrease in locomotor activity on all days tested, whereas AAVantiNic-treated mice showed the same ambulatory activity profile as the control mice given saline instead of nicotine (Fig. 5A). At day 18 of the nicotine challenge study (7 weeks after administration of AAVantiNic), we measured the cumulative distance traveled after nicotine administration. Naïve control mice exhibited a nicotine-induced suppression of ambulatory activity (2.15 ± 0.30 m over 15 min, $P < 0.003$) compared to saline-injected control mice, whereas AAVantiNic mice showed no nicotine-induced reduction in locomotor activity (6.38 ± 1.20 m), similar to control mice given saline (5.00 ± 0.75 m; $P > 0.6$; Fig. 5B). The cumulative vertical activity profile demonstrated similar AAVantiNic-mediated protection from nicotine. Control mice given nicotine displayed 16.8 ± 3.0 s (over 15 min) of vertical activity ($P < 10^{-5}$ compared to saline-injected control mice), and AAVantiNic mice given nicotine exhibited 180.2 ± 26.7 s of vertical activity, similar to the 200.6 ± 36.6 s seen in the control mice given saline instead of nicotine ($P > 0.9$; Fig. 5C).

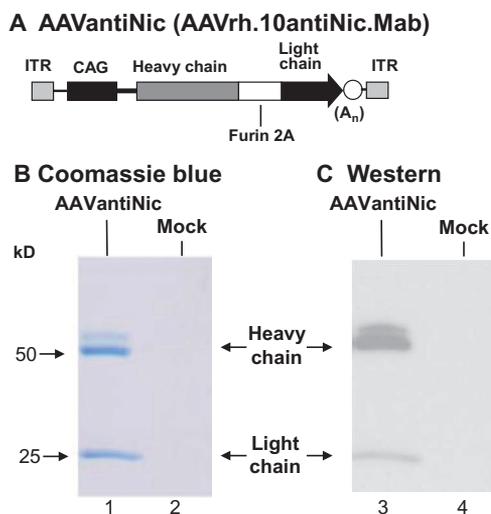
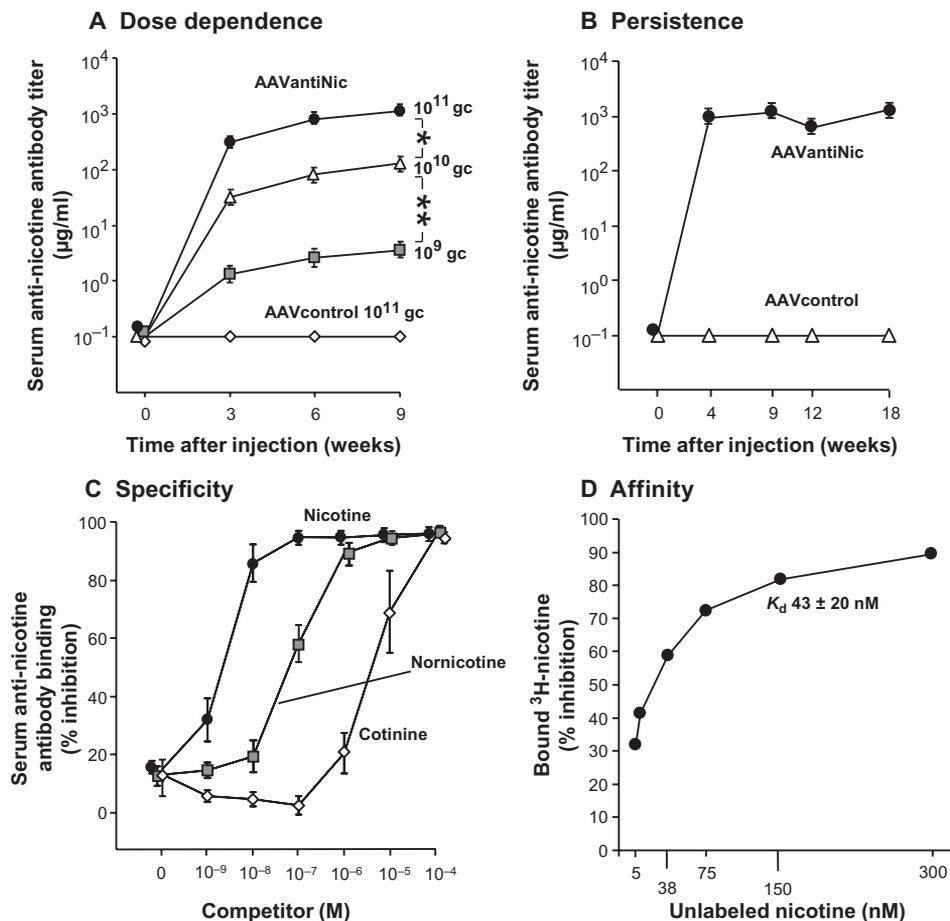


Fig. 1. Structure and function of the AAVantiNic gene transfer vector. (A) Schematic of the AAVantiNic vector with CMV enhancer/chicken β -actin promoter (CAG) promoter, heavy and light chain of the anti-nicotine monoclonal NIC9D9, Furin 2A polypeptide cleavage site, and polyadenylation signal. (B and C) AAVantiNic-directed expression and secretion of the anti-nicotine monoclonal antibody NIC9D9. HEK 293 cells were infected with 2×10^5 gc per cell of AAVantiNic or mock-infected. After 72 hours, the supernatant was harvested and the antibody was purified by protein G-Sepharose. (B) Coomassie blue-stained, reducing SDS-PAGE analysis of purified IgG from cell supernatant. Lane 1, AAVantiNic; lane 2, mock-infected. (C) Anti-IgG Western analysis of purified IgG from cell supernatant. Lane 3, AAVantiNic; lane 4, mock-infected.

DISCUSSION

The challenge of developing a successful vaccine for nicotine addiction is that it must evoke a high-titer, high-affinity, specific antibody in the broad spectrum of genetic backgrounds found in humans, a hurdle difficult to overcome through active vaccination. Our approach avoids this obstacle by using the AAVrh.10 gene expression vector to effectively and systemically deliver the anti-nicotine monoclonal antibody NIC9D9 to mice. Our previous studies in which we measured the expression of a reporter transgene (α 1-antitrypsin) from intravenously delivered AAVrh.10 vectors in mice show that the vector achieves very high transduction efficiency in the liver, with only minor transduction in other organs (25). Here, we have extended this approach and shown that an AAVantiNic vector expresses a full-length, high-affinity anti-nicotine monoclonal antibody and that a single

Fig. 2. AAVantiNic-directed expression of an anti-nicotine antibody in mice. **(A)** Anti-nicotine IgG antibody titers at 10^9 , 10^{10} , and 10^{11} gc of AAVantiNic or of 10^{11} gc of AAVcontrol administered intravenously to C57BL/6 mice ($n = 5$ for each condition). Antibody titers were assessed by ELISA against bovine serum albumin (BSA)-conjugated nicotine hapten (AM1) ($*P < 0.005$, $**P < 0.0003$). **(B)** Persistence of expression of the anti-nicotine antibody for 18 weeks after administration of AAVantiNic. C57BL/6 mice ($n = 10$ each condition) were injected intravenously with 10^{11} gc of AAVantiNic or AAVcontrol. For (A) and (B), antibody titers were calibrated with a NIC9D9 antibody standard. **(C)** Specificity of anti-nicotine antibody derived from AAVantiNic. C57BL/6 mice were given 10^{11} gc of AAVantiNic intravenously, and serum was collected at 12 weeks. Competitive inhibition of binding of BSA-AM1 in sera was measured by incubation with increasing concentrations of nicotine, nornicotine, or cotinine in the ELISA ($n = 4$). **(D)** Affinity (K_d) of the anti-nicotine antibody derived from AAVantiNic. The serum from (C) was used to determine the K_d by competitive inhibition of tracer ($[^3H]$ nicotine) binding with unlabeled nicotine. K_d value is derived from three independent experiments and expressed as mean \pm SEM.



administration of AAVantiNic to mice results in persistent, high concentrations of the antibody in serum at a 20-fold molar excess over the serum nicotine concentration of 70 to 110 ng/ml (greater than the nicotine serum concentrations of a continuous smoker) (11, 26, 27). In mice treated in this way with AAVantiNic, parenterally administered nicotine became bound to antibody and was sequestered in the blood, preventing the drug from reaching the central nervous system, even when the animal was challenged with a dose higher than that seen in chronic smokers. Mice expressing the vector-derived antibody failed to respond to nicotine with the usual alterations in cardiovascular function or in ambulatory and vertical locomotor activity. If, in additional experiments, AAVantiNic can interfere with nicotine self-administration in a rodent model of human addiction, this therapeutic approach will be a good candidate for human clinical studies.

Previous studies with active anti-nicotine vaccines have attempted to generate a potent humoral immune response against nicotine. The challenge of this approach is that nicotine is a small nonimmunogenic molecule, as demonstrated by the absence of natural immunity in smokers. To impart immunopotency, nicotine (or a nicotine analog) must be coupled to a larger molecule to induce a sufficient anti-nicotine immune response (20, 28, 29). For example, AM1, a *trans*-3'-(hydroxymethyl)nicotine-derived hapten with a linker containing an ether moiety and a free carboxyl group for conjugation, has been attached to carriers such as tetanus toxin to create an anti-nicotine vaccine. In a rodent self-administration model, this vaccine increased

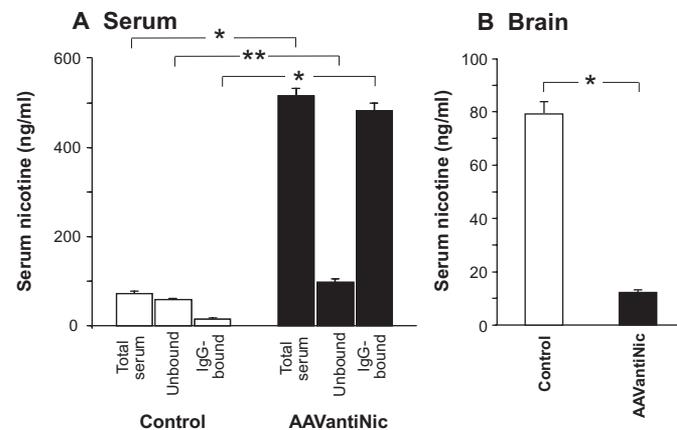


Fig. 3. Nicotine accumulation in serum and brain of C57BL/6 mice. $[^3H]$ Nicotine was administered intravenously to naive control and AAVantiNic-treated mice ($n = 3$) 15 weeks after treatment, and 1 min later, nicotine was measured in the serum (ng/ml) and brain (ng/g). **(A)** Serum nicotine concentrations. IgG-bound and free $[^3H]$ nicotine was assessed ($*P < 10^{-5}$, $**P < 0.005$). **(B)** Brain nicotine concentrations. Comparisons between groups were conducted by a two-tailed unpaired *t* test ($*P < 10^{-4}$).

motivation for nicotine self-administration (30), in effect causing the animal to surmount vaccine-mediated blockade of nicotine action. Three active immunotherapy vaccines have been in clinical

trials, including TA-NIC [a nicotine analog linked to cholera toxin B (Celtic Pharma)], NicVAX [a nicotine analog linked to *Pseudomonas aeruginosa* exoprotein A (Nabi Pharmaceuticals)], and NicQb [a nicotine analog linked to particles of the bacteriophage Q β (Cytos Biotechnology)] (17, 31). The carriers for NicVAX and NicQb are conjugated to 3'-aminomethyl nicotine, whereas the TA-NIC carrier is conjugated to an *N*'-butyric acid adduct of (s) nicotine (17, 30, 32). The data that are publicly available indicate that these vaccines are well tolerated, and the individuals with the highest circulating concentrations of antibodies are most likely to abstain from smok-

ing (17). However, in all cases, there has been a large variation among the trial participants in the amount of antibody generated, and only a relatively small percentage of the participants—those with the highest serum anti-nicotine antibody titers (24.6% versus 12.0%

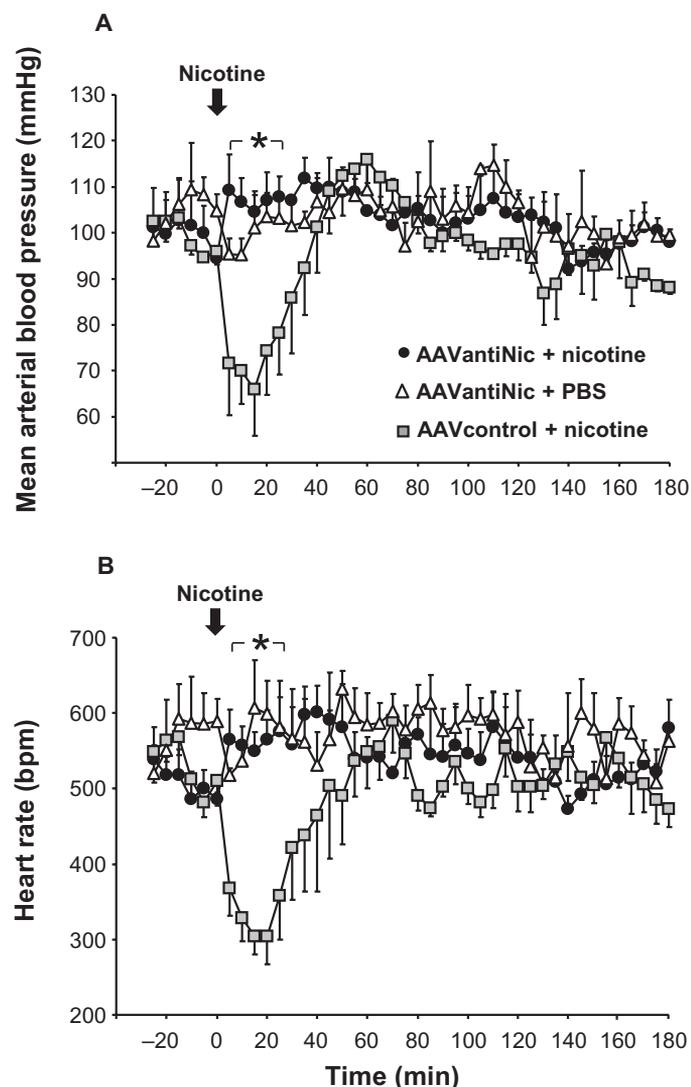


Fig. 4. Prevention of nicotine-induced cardiovascular effects by AAVantiNic. Seven weeks after AAVantiNic or AAVcontrol treatment, radiotelemetry devices were implanted in mice, and 1 week later, 30 min of baseline mean arterial blood pressure and heart rate were assessed. (A and B) Mice were then nicotine-challenged and monitored for 180 min for (A) mean arterial blood pressure and (B) heart rate. AAVantiNic abolished nicotine-induced mean arterial blood pressure depressor and heart rate bradycardic responses during the first 25 min ($*P < 10^{-3}$). Comparisons between groups were conducted by two-way repeated-measures ANOVA. bpm, beats per minute.

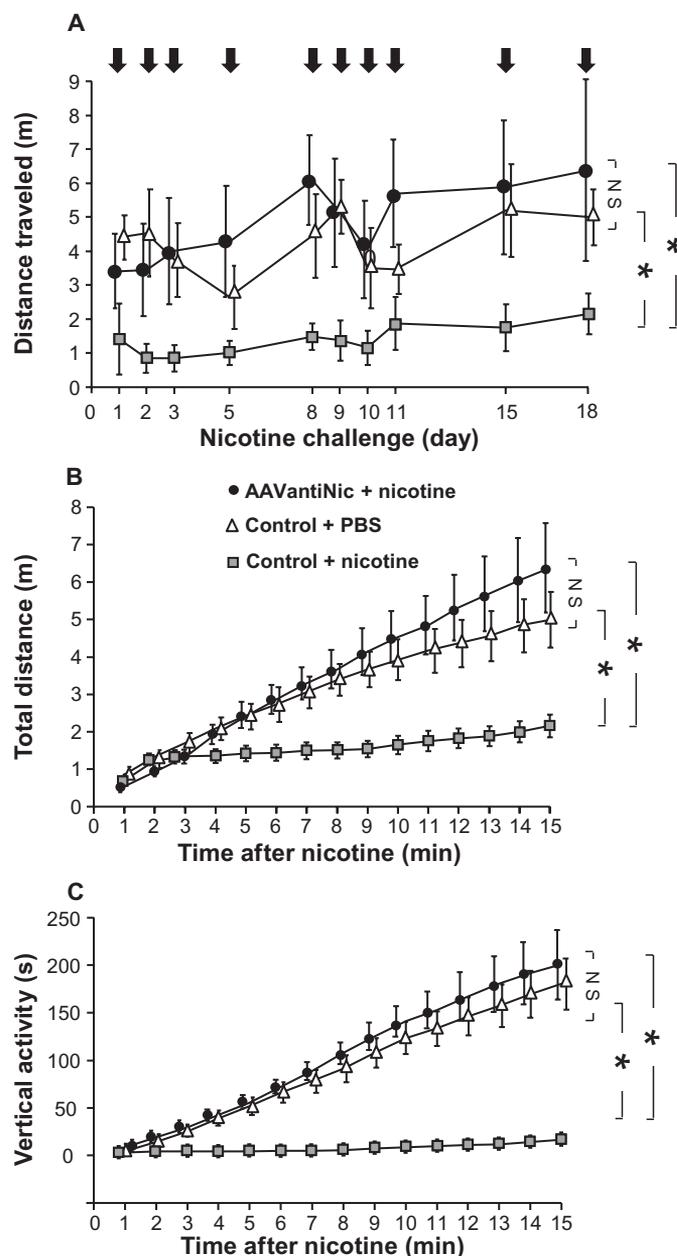


Fig. 5. AAVantiNic-mediated prevention of nicotine-induced hypolocomotor activity. (A) Total distance traveled of naïve control and AAVantiNic-treated mice over time with frequent nicotine or PBS challenges (arrows). The mice were treated with a single administration of AAVantiNic 7 weeks before the last challenge [not significant (NS) $P > 0.1$, $*P < 10^{-5}$]. (B) Cumulative distance traveled as a function of time after administration of PBS or nicotine. The data are from day 18, the longest time point evaluated, 7 weeks after treatment (NS $P > 0.7$, $*P < 10^{-3}$). (C) Cumulative vertical activity as a function of time after administration of PBS or nicotine in the same mice as in (B) (NS $P > 0.9$, $*P < 10^{-4}$).

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Table 1. Comparison of AAVantiNic to other anti-nicotine vaccines. Data are expressed as means \pm SD.

Parameter	NicVAX ^{††}	NicQb ^{‡§}	TA-NIC [¶]	AAVantiNic [§]
Administration schedule (days)	0, 21, and 42	0 and 28	0, 14, and 28	0
Day of titer evaluation	49	54	35	126
Antibody concentration (μ g/ml)	280 \pm 120	163.5	N/A [¶]	1310 \pm 230
K _d (nM)	10 to 40	46 \pm 19	56	43 \pm 20
Brain reduction (%) ^{††}	66 (at 3 min)	57 (at 5 min)	85 (at 5 min)	85 (at 1 min)

*NicVAX by Nabi Pharmaceuticals is a nicotine analog linked to *P. aeruginosa* exoprotein A formulated in rats with Freund's adjuvant (35). †Study in rats (34, 35). ‡NicQb by Cytos Biotechnology is a nicotine analog linked to particles of bacteriophage Q β and formulated on alum (29). §Study in mice (27, 46). ¶TA-NIC by Celtic Pharma is a nicotine analog coupled to cholera toxin B and formulated on alum (46). ¶¶Not applicable, antibody not calibrated to protein concentration. ††Percent reduction in brain nicotine content after administration of [³H]nicotine (0.03 mg/kg, intravenously).

in the placebo group of the NicVAX phase 2b clinical trial)—have abstained from smoking (17, 31–33). Our vector, AAVantiNic, expresses an anti-nicotine antibody with high affinity that is similar to that of the investigational vaccines NicVAX (34, 35) and NicQb (29) but with a much higher concentration in the serum. This antibody effectively prevents nicotine from entering the brain at only 1 min after administration, suggesting that AAVantiNic is a more effective delivery tool, with a therapeutic threshold at higher doses of administered nicotine (Table 1).

In addition to causing nicotine addiction, cigarette smoking is strongly linked to the development of a number of pathological conditions, including diseases of the cardiovascular system (5). Central neural pathways, in part, mediate nicotine's acute and chronic effects on cardiovascular control (36), although the mechanisms remain largely undefined. In line with previous findings (37, 38), we demonstrate a significant bradycardia and blood pressure reduction in response to parenteral nicotine administration that results in a serum concentration higher than that of a typical smoker. This response is completely abolished in AAVantiNic-treated mice, showing that AAVantiNic can also abrogate nicotine-mediated physiological changes, likely including the adverse cardiovascular effects associated with cigarette smoking (which may also play a role in drug reinforcement beyond the neurological chemical addiction) (39).

Our results suggest that clinical translation of AAVantiNic is appropriate. We have completed safety studies for two AAVrh.10 vectors (carrying different transgenes), one of which is being used in an ongoing clinical trial for Batten disease in children (ClinicalTrials.gov, NCT01161576). Immunotherapy with the AAV vector-directed expression of monoclonal antibodies as a treatment for drug addiction offers a unique opportunity to address a great societal problem. Unlike current smoking cessation therapies, which require daily treatment and lead to noncompliance, this approach would circumvent this issue because it requires only a single administration.

MATERIALS AND METHODS

Recombinant AAVrh.10 vectors

AAVantiNic vector is based on the nonhuman primate-derived rh.10 capsid pseudotyped with AAV2 inverted terminal repeats (ITRs) surrounding the anti-nicotine antibody expression cassette (Fig. 1A). The expression cassette consists of cytomegalovirus (CMV) enhancer/chicken β -actin promoter, the anti-nicotine monoclonal NIC9D9 heavy chain coding sequence, a 4-amino acid furin cleavage site, the 24-amino

acid self-cleaving 2A peptide, the anti-nicotine monoclonal NIC9D9 light chain coding sequence, and the rabbit β -globin polyadenylation signal (22, 23, 40–42). The complementary DNA (cDNA) sequence of the NIC9D9 antibody heavy chain (IgG1) and light chain (κ chain) was cloned from the mouse hybridoma NIC9D9 (42) with RNA ligase-mediated rapid amplification of cDNA ends (GeneRacer kit; Invitrogen) with mouse Ig gene-specific primers. The negative control vector AAVrh.10antiPA.Mab (referred to as AAVcontrol) encoded an irrelevant antibody directed against anthrax protective antigen.

AAVantiNic was produced by PolyFect-mediated (Qiagen) cotransfection into HEK 293 (American Type Culture Collection) of three plasmids, pAAV α NIC9D9 (600 μ g), pAAV442 (600 μ g), and pAd Δ F6 (1.2 mg): (i) pAAVNIC9D9 is an expression plasmid containing (5' to 3') the AAV2 5' ITR including packaging signal (ψ), the anti-mouse NIC9D9 antibody expression cassette, and the AAV2 3' ITR; (ii) pAAV44.2 is a packaging plasmid that provides the AAV Rep proteins derived from AAV2 needed for vector replication and the AAVrh.10 viral structural (Cap) proteins VP1, 2, and 3, which define the serotype of the produced AAV vector; and (iii) pAd Δ F6 is an Ad helper plasmid that provides Ad helper functions of E2, E4, and V_A RNA (43). At 72 hours after transfection, the cells were harvested, and a crude viral lysate was prepared by four cycles of freeze/thaw and clarified by centrifugation. AAVantiNic was purified by iodoxanol gradient and QHP anion exchange chromatography. The purified AAVantiNic was concentrated with an Amicon Ultra-15 100K centrifugal filter device (Millipore) and stored in PBS (pH 7.4) at -80°C . The control vector was produced by this method with pAAVantiPA.Mab substituted for pAAVantiNic. Vector genome titers were determined by quantitative TaqMan real-time polymerase chain reaction analysis with a CMV promoter-specific primer-probe set (Applied Biosystems).

In vitro assessment of AAVantiNic expression

To assess AAVantiNic-directed expression of the monoclonal antibody in vitro, we infected HEK 293 cells with AAVantiNic at 2×10^5 gc per cell (or mock-infected), harvested supernatant 72 hours later, and purified Ig with protein G-Sepharose. Anti-nicotine antibody expression was evaluated by Coomassie blue-stained SDS-PAGE and Western analysis (41) with a sheep anti-mouse IgG heavy chain and light chain secondary antibody (Sigma) and enhanced chemiluminescence reagent (Amersham).

Intravenous AAVantiNic delivery

All animal studies were conducted under protocols reviewed and approved by the Weill Cornell Institutional Animal Care and Use Committee. Male C57BL/6 mice, 4 to 6 weeks old (Taconic), were housed

under pathogen-free conditions. At 7 to 9 weeks of age, the mice were treated with AAVantiNic at 10^9 , 10^{10} , or 10^{11} gc by intravenous injection in 100- μ l volume.

Evaluation of anti-nicotine antibody titer

Blood was obtained by drawing 250 μ l of blood from the tail vein at time 0 and at various time points, until 18 weeks. The blood samples were allowed to clot for 1 hour at 23°C, followed by 30 min at 4°C, and then spun in an Eppendorf microcentrifuge at 10,000 rpm for 20 min to collect serum. The concentration of anti-nicotine antibody was then determined by ELISA. Wells of flat-bottomed 96-well EIA/RIA plates (Corning) were coated with 100 μ l of bovine serum albumin (BSA) (1 mg/ml) conjugated with AM1, a *trans*-3'-(hydroxymethyl)nicotine-derived nicotine hapten (30) (ratio of 2:1), in carbonate buffer overnight at 4°C and then washed with 0.05% Tween 20 in PBS (PBS-Tween) and blocked with 5% dry milk in PBS for 30 min at 23°C. Serial dilutions of sera were incubated for 90 min at 23°C. The plates were washed four times with PBS-Tween and 100 μ l of 1:2000 diluted goat anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz) in 1% dry milk in PBS and incubated for 90 min at 23°C. After four wash steps, peroxidase substrate (100 μ l per well; Bio-Rad) was added to each well and incubated for 15 min at 23°C, and the reaction was stopped with addition of 2% oxalic acid (100 μ l per well). Absorbance was measured at 415 nm. Anti-nicotine antibody titers were calculated by interpolation of the $\log(\text{OD}) - \log(\text{dilution})$ with a cutoff value equal to twice the absorbance of background and converted to micrograms per milliliter on the basis of standard curve with the NIC9D9 antibody. The NIC9D9 antibody was quantified by the bicinchoninic acid assay (Pierce Biotechnology). Antibody specificity for nicotine and metabolites was evaluated by competitive ELISA with sera from AAVantiNic-treated mice in BSA-AM1 in the presence of increasing concentrations of nicotine and nicotine metabolites nor-nicotine or cotinine, from 0.1 nM to 0.1 mM. Affinity of the expressed antibody from sera from AAVantiNic-treated mice was evaluated by a radioimmunoassay, using [^3H]nicotine (10 nm) with increasing concentrations of unlabeled nicotine (1 to 300 nm) as the unlabeled competitor (44).

Nicotine accumulation

Naïve control or AAVantiNic-treated mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) 3 min before intravenous administration of 0.8 μ g of nicotine (equivalent to a two-cigarette human dose) containing 1.0 μ Ci of [^3H]nicotine (11, 24). One minute later, mice were sacrificed, and the brain and blood were collected separately. Brain tissue was homogenized in PBS, and 300 μ l of brain homogenate and separately 50 μ l of serum were added to 5 ml of liquid scintillant (Ultima Gold, PerkinElmer), which was assessed for radioactivity, and the data were normalized with a standard quenching curve. For the blood compartment, nicotine was normalized to serum volume (ng/ml), and brain nicotine was normalized to brain wet weight (ng/g).

Nicotine-induced cardiovascular parameters

To assess whether the treatment abrogated the cardiovascular effects of nicotine, we implanted mice with radiotelemeters (Data Sciences International, PA-C10 model) 7 weeks after administration of AAVantiNic or AAVcontrol vector as described (45). One week after telemeter implantation, baseline measurements of mean arterial blood pressure and

heart rate were collected for 30 min in conscious mice. Subsequently, nicotine (25.0 μ g, 1.0 mg/kg) or PBS was administered subcutaneously, and cardiovascular parameters were recorded for 180 min with the DSI Dataquest A.R.T. System (Data Sciences International).

Nicotine-induced locomotor behavior

We recorded mouse locomotor behavior with infrared beam-equipped activity chambers (20-cm by 20-cm chamber, AccuScan Instruments) at 4 weeks after AAVantiNic treatment. Mice were allowed to habituate to the room for 1 hour before each test. Mice were placed in the chamber for 15 min to record prechallenge behavior and then removed, injected with PBS or nicotine (12.5 μ g, 0.5 mg/kg) subcutaneously, and returned to the chamber for 15 min. Locomotor assays were repeated over a 3-week period for a total of 10 nicotine challenges. Chambers recorded ambulatory distance traveled and vertical movement.

Statistics

Dose-response and pharmacokinetic data are expressed as means \pm SEM, and comparisons between groups were conducted by a two-tailed unpaired *t* test. For cardiovascular studies, mean arterial blood pressure and heart rate time point data over the 210-min course were compared by two-way repeated-measures analysis of variance (ANOVA) with AAVantiNic treatment as the between-subjects variable and time as the within-subjects variable, and Bonferroni post hoc comparison was performed at each time point. The two-way repeated-measures ANOVA was used for locomotor studies with AAVantiNic treatment as the between-subjects variable and nicotine challenge day as the within-subjects variable. Comparisons of cumulative distance and vertical activity between groups were performed with the Kolmogorov-Smirnov test.

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