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Combined Active and Passive Immunization Enhances the Efficacy of Immunotherapy against Nicotine in Rats

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ABSTRACT

Vaccination against nicotine reduces the behavioral effects of nicotine in rats, and it is under clinical evaluation as a treatment for tobacco addiction. Efficacy is limited by the need for high serum nicotine-specific antibody (NicAb) levels, and currently available nicotine vaccines do not uniformly generate the required NicAb levels. Passive immunization with a nicotine-specific monoclonal antibody (Nic311) has also shown efficacy in rats. The principal aim of this study was to determine whether the combined use of vaccination and passive immunization would produce greater effects than vaccination alone on nicotine pharmacokinetics and locomotor sensitization (LMS) to nicotine. Rats were treated with vaccination alone, Nic311 alone, both, or neither, and then they were administered 10 daily injections of 0.3 mg/kg nicotine s.c. Treatment with Nic311 or vaccination alone increased the binding of nicotine in serum, reduced the unbound serum nicotine concentration and nicotine distribution to brain, and attenuated the development of LMS. Combined use of vaccination and passive immunization produced higher total serum NicAb levels, greater changes in nicotine pharmacokinetics, and a greater attenuation of LMS than either treatment alone. The total serum NicAb concentration was significantly correlated with brain nicotine levels and locomotor activity. These data indicate that providing higher serum NicAb concentrations improves the efficacy of immunotherapy against nicotine and that supplementing vaccination with passive immunization is a potential strategy to accomplish this.

Cigarette smoking is the leading preventable cause of death in the United States (Mokdad et al., 2004). Nicotine is the principle addictive component of tobacco smoke (Benowitz, 1996), and the rapid absorption and distribution of nicotine from cigarette smoke into the brain produces reinforcing effects that help initiate and maintain tobacco dependence (Henningfield and Keenan, 1993). Interventions that suppress or slow the distribution of nicotine to brain may be useful to treat tobacco dependence.

Immunization against nicotine is being studied as a means of altering the pharmacokinetics of nicotine, and it has substantial effects in animal models of nicotine addiction. Immunization of rats generates nicotine-specific antibodies (NicAbs) that bind nicotine in serum, reduce the unbound concentration of nicotine, and reduce nicotine distribution to brain (Hieda et al., 1999; Pentel et al., 2000; Satoskar et al., 2003; de Villiers et al., 2004; Cerny, 2005; Maurer et al., 2005). Vaccination against nicotine attenuates a wide range of behavioral effects of nicotine relevant to addiction, including the acquisition, maintenance, and reinstatement of nicotine self-administration (Lindblom et al., 2002; LeSage et al., 2006a). Three nicotine vaccines are currently in clinical trials, and preliminary data indicate efficacy for enhancing smoking cessation rates (Hatsukami et al., 2005; Maurer and Bachmann, 2007; C. Bunce and A. Fattom, personal communication).

Although this approach seems to be promising, a major limitation of vaccination against nicotine as a strategy for treating tobacco addiction is the need to reliably generate high serum NicAb levels. In rats, the effects of immunization

ABBREVIATIONS: NicAb, nicotine-specific antibody; Nic311, nicotine-specific monoclonal antibody 311; LMS, locomotor sensitization; 3′-AmNic, 3′-aminomethyl nicotine; rEPA, recombinant *Pseudomonas* exoprotein A; 3′-AmNic-rEPA, 3′-aminomethyl nicotine conjugated to recombinant *Pseudomonas* exoprotein A; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; ANOVA, analysis of variance.

on nicotine pharmacokinetics are strongly correlated with the serum NicAb levels achieved; higher serum NicAb concentrations are associated with greater binding of nicotine in serum and reduced distribution of nicotine to brain (Hieda et al., 1999; Keyler et al., 2005b; Maurer et al., 2005). Enhanced smoking cessation rates have been reported in clinical trials of nicotine vaccines, but they are confined to those subjects with the highest serum antibody concentrations (Hatsukami et al., 2005; Maurer and Bachmann, 2007; C. Bunce and A. Fattom, personal communication). Two limitations of vaccination are apparent from clinical trials: the mean serum NicAb concentrations are lower than those reported in rats or mice (Pentel et al., 2000; Keyler et al., 2006), and there is substantial variability (up to a 30-fold range) in those concentrations (Hatsukami et al., 2005; Maurer and Bachmann, 2007). Developing strategies to reliably produce high serum antibody concentrations in response to nicotine vaccines is critical to maximizing their efficacy.

Immunization against nicotine can be accomplished via either active immunization (vaccination) or passive immunization (administration of preformed antibodies). Initial efforts to develop immunotherapies against nicotine have used vaccination because of its long-lasting effects, reasonable cost, and the excellent safety record of vaccines in general. Passive immunization with nicotine-specific monoclonal antibodies has also been studied in rats, and it produces effects on nicotine pharmacokinetics that are similar to those of vaccination (Pentel et al., 2000, 2006; Carrera et al., 2004; Keyler et al., 2005b). Effects of passive immunization on nicotine-related behaviors are less well studied, but attenuation of nicotine-induced locomotor activity has been reported previously (Pentel et al., 2000; Carrera et al., 2004). Passive immunization has potential advantages over vaccination in that higher doses of antibody can be administered than can be elicited by vaccination, the antibody dose can be controlled so that individual variability in serum antibody levels might be minimized, and the onset of effect is immediate. For these reasons, passive immunization is of interest as a possible therapeutic alternative to vaccination, and it is also being studied for the treatment of methamphetamine, phencyclidine, and cocaine addictions (Daniels et al., 2006; Pitas et al., 2006; Norman et al., 2007). The main disadvantage of passive immunization is that it is considerably more expensive than vaccination. Nevertheless, passive immunization with various monoclonal antibodies, in some cases at cumulative doses comparable with those used in the current study, is now widely used to treat cancer, inflammatory diseases, and other disorders (Tabrizi and Roskos, 2007).

In the current study, the effects of vaccination, passive immunization, or a combination of the two were studied with regard to their effects on serum NicAb levels, nicotine pharmacokinetics, and locomotor sensitization (LMS) to nicotine. The purposes of this study were to 1) compare the effects of vaccination with those of passive immunization, 2) assess whether providing serum NicAb levels higher than those achieved by vaccination alone would further improve efficacy, and 3) evaluate the feasibility of using the combination of vaccination and passive immunization as a general strategy for enhancing the efficacy of immunotherapy for nicotine addiction. The pharmacokinetics of the nicotine-specific monoclonal antibody Nic311 was also characterized to guide Nic311 dosing.

Materials and Methods

General Methods

Animals. Male Holtzman Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 275 to 300 g, were housed individually in temperature- and humidity-controlled colony rooms with unlimited access to water under a 12-h light/dark cycle (lights off at 10:00 PM). Rats were food-restricted to 18 g/day rat chow to minimize weight gain and chronic catheter displacement throughout the chronic studies. All protocols were approved by the Minneapolis Medical Research Foundation Animal Care and Use Committee, and they were in accordance with National Institutes of Health guidelines.

Reagents. Nicotine bitartrate (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile heparinized saline (30 units/ml). The pH of the solution was adjusted to 7.4 with dilute NaOH. All nicotine doses and concentrations are expressed as that of the base.

The nicotine immunogen used in this study is a well characterized conjugate consisting of the hapten 3′-aminomethyl nicotine (3′-AmNic) linked to the carrier protein recombinant *Pseudomonas* exoprotein A (rEPA) (Pentel et al., 2000). This immunogen (3′-AmNic-rEPA) generates antibodies in rats that have a high affinity for nicotine (K_d = 19–40 nM) and <1% cross-reactivity with acetylcholine, the major nicotine metabolites cotinine and nicotine-N-oxide, and a variety of other neurotransmitters or medications (Pentel et al., 2000). Control immunogen was the unconjugated rEPA alone. Vaccine doses consisted of 25 μg of immunogen in complete Freund's adjuvant for the first dose and in incomplete Freund's adjuvant for subsequent booster doses. The nicotine-specific monoclonal antibody used in this study (Nic311) is an IgG $1_κ$ isoform derived from mice immunized with 3′-AmNic-rEPA, with a K_d value for nicotine of 60 nM. Nic311 was purified by protein G chromatography to ≥95% of total protein content with endotoxin levels of <0.2 enzyme unit/mg. Nic311 was diluted in phosphate-buffered saline to a concentration of 10 mg/ml for administering doses of 30 mg/kg in the pharmacokinetic experiment and 30 mg/ml for doses of 80 mg/kg in the locomotor experiment. Control IgG was human polyclonal IgG (Sandoglobulin; Sandoz, Vienna, Austria) that does not bind nicotine or alter nicotine pharmacokinetics or behavior in rats (Keyler et al., 2005b). Nic311 has <1% cross-reactivity with nicotine metabolites or acetylcholine (Pentel et al., 2006).

Nicotine and NicAb Assays. Serum and brain nicotine levels were measured by gas chromatography with nitrogen-phosphorous detection (Jacob et al., 1981). Brain nicotine concentrations were corrected for brain blood content (Hieda et al., 1999).

Nicotine-specific antibody concentrations generated by vaccine were measured by ELISA using 3′-AmNic-polyglutamate as the coating antigen and goat anti-rat IgG-horseradish peroxidase (HRP) as detecting antibody. The same coating antigen was used to measure Nic311 concentrations, and the detecting antibody was goat anti-mouse-IgG-HRP. Because Nic311 was administered to one group of rats that had already been vaccinated, it was necessary to determine cross-reactivity of the antibodies generated by vaccination in the ELISA used to quantitate Nic311 levels. This was accomplished by assaying serum samples using both the rat and mouse quantitative ELISAs. All reported Nic311 levels were corrected for this cross-reactivity. In contrast, serum nicotine-specific antibody levels generated by vaccination were measured before Nic311 administration, so no correction of these values was required.

ELISA to Detect Antibodies against Nic311. The purpose of this assay was to detect the potential presence of rat anti-mouse antibodies in the Nic311 pharmacokinetic parameters experiment. Plates were coated with 1 ng of Nic311 per well. Serum samples from days 8 to 35 were added to wells, samples were serially diluted, and the plate was incubated overnight at 4°C. Detecting antibody was goat anti-rat IgG or horse anti-rat IgM conjugated to HRP.

Protein Binding. Equilibrium dialysis of serum was performed in 1.0-ml Teflon cells with Spectra/Por 2 membranes (Spectrapor

Labs, Rancho Dominguez, CA) as described previously (Pentel and Keyler, 1988). The fraction unbound was the ratio of the nicotine concentrations in dialysate and serum. Unbound nicotine concentration was calculated as the product of the total serum nicotine concentration before dialysis and the fraction unbound.

Nic311 Pharmacokinetic Parameter Estimates

Two groups of six to seven rats were anesthetized with intramuscular 2.0 mg/kg droperidol and 0.04 mg/kg fentanyl, and cannulae were inserted into the jugular and femoral veins, externalized between the scapulae, and attached to a guide cannula mounted in a harness assembly (Instech, Plymouth Meeting, PA). A stainless steel spring tether attached to the guide cannula allowed connection to a fluid swivel for antibody administration and sample collection. The femoral cannula was used to administer Nic311, and the jugular cannula was used for sampling.

Rats were administered a single dose of 30 mg/kg i.v. Nic311 in a volume of 1 ml via the femoral cannula. One group of rats was implanted with a s.c. osmotic minipump (Alzet 2ML4; DURECT Corporation, Cupertino, CA) delivering 3.2 mg/kg/day nicotine to determine Nic311 pharmacokinetics in the presence of nicotine, because nicotine would be present in the LMS protocol (see below). The minipump was replaced on day 28. Blood samples (0.5 ml) were collected from the jugular cannula at intervals for 56 days (more than five terminal half-lives), and serum was stored at $-20°C$ until analyzed for nicotine content as described above. Pharmacokinetic parameters were estimated by two-compartment analysis using WinNonlin 4.0 (Pharsight, Mountain View, CA).

Locomotor Sensitization to Nicotine

Protocol. Five groups of rats ($n = 9–15$/group) were immunized with 3′-AmNic-rEPA or control vaccine on days 1, 21, 42, and 59 (Fig. 1, see timeline). On days 62 to 64, a chronic jugular cannula was implanted, and a serum sample was taken to measure vaccine-generated nicotine-specific antibodies. On day 69, rats began the nicotine sensitization protocol of 10 daily doses of saline or nicotine 0.3 mg/kg s.c. Rats were not exposed to the testing chambers before beginning the sensitization protocol. Locomotor activity (horizontal distance traveled) was measured for 30 min immediately following each nicotine dose. One hour before the first and the seventh session, rats were administered Nic311 or control IgG via the jugular cannula. The Nic311 doses of 80 mg/kg on day 1 and 40 mg/kg on day 7 were based on the terminal half-life of Nic311 in the presence of nicotine as determined above (7.6 days) (Table 2), and they were intended to maintain serum Nic311 concentrations equal to those expected from vaccination (approximately 200 µg/ml) throughout the locomotor sensitization period. Group treatments are shown in Table 1. Immediately after the final (10th) locomotor session (40 min after the nicotine dose), all animals were administered a terminal dose of pentobarbital, 50 mg/kg, to confirm catheter patency. They were immediately sacrificed, and brain and serum samples were collected for analysis. Data from animals with catheters that had failed ($n = 3$) were not used.
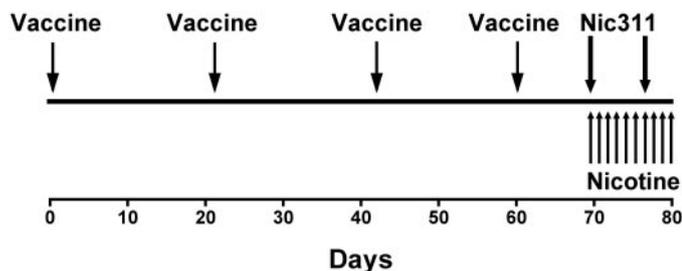

Fig. 1. Timeline for the locomotor sensitization experiment. Locomotor activity was measured after each daily dose of 0.3 mg/kg nicotine or saline s.c.

Group	Nicotine	Vaccine Immunogen	Passive Immunization
Nicotine control	+	rEPA	Control-IgG
Vaccine	+	3′-AmNic-rEPA	Control-IgG
Nic311	+	rEPA	Nic311
Combined	+	3′-AmNic-rEPA	Nic311
Saline control	−	rEPA	Control-IgG

Locomotor Apparatus. Locomotor monitoring sessions were conducted in open field activity chambers (MED Associates, St. Albans, VT) measuring 43×43 cm. Each chamber had two 16-beam photocell arrays placed 2.5 cm and one array 8 cm above the chamber floor to monitor horizontal and vertical activity. Chambers were placed inside sound-attenuating cubicles that had exhaust fans providing masking noise and ambient lighting. A computer with open-field activity software (MED Associates) was used for operating the apparatus and recording data.

Statistical Analyses

Nic311 pharmacokinetic parameters in the presence and absence of nicotine were compared by Wilcoxon's rank sum test. This nonparametric test was used because of small group sizes. Serum NicAb concentrations, serum and brain nicotine levels, free nicotine levels, and percentage of bound were analyzed by Kruskal-Wallis tests, with the Wilcoxon rank sum test for multiple comparisons and Bonferroni's post-tests. This nonparametric test was used because of unequal group variances.

Locomotor activity was measured as total horizontal distance traveled over the 30-min session. Locomotor activity analysis consisted of an overall two-way ANOVA with group as a between-subjects factor and day as a within-subjects factor. Subsequent one-way ANOVAs were used to analyze within-group distance traveled across days, with Dunnett's post-test to compare day 1 to the subsequent days. In addition, a one-way ANOVA was used to examine locomotor activity between groups on day 1.

Because the nicotine group exhibited significant sensitization on days 7 to 10 compared with day 1, data from these days were used to analyze treatment effects on locomotor activity between groups. Distance traveled across days 7 to 10 was analyzed using two-factor ANOVA, with group as a between-subjects factor and day as a within-subjects factor, followed by Bonferroni's post-test to compare marginal means between groups. Because most locomotor activity occurs during the beginning of the locomotor activity sessions, a second planned comparison was analysis of within session data on days 1 and 10. Within-session data were separated into 5-min blocks, and day 1 and day 10 were analyzed separately using two-factor ANOVA, with group as a between-subjects factor and time as a within-subjects factor, followed by Bonferroni's post-test to compare mean activity scores between groups at each 5-min bin.

The relationships between total serum NicAb concentrations and free serum nicotine levels, brain nicotine levels, and mean locomotor activity across days 7 to 10 were analyzed using linear regression analysis. Relationships between free serum nicotine and brain nicotine levels, and brain nicotine levels and locomotor activity were similarly analyzed.

Inspection of the serum NicAb concentration versus locomotor activity data (Fig. 7, bottom) suggested that a serum NicAb concentration of ≥300 µg/ml was associated with maximal efficacy in the attenuation of locomotor activity (i.e., activity comparable with that of saline controls). To examine this further, a t test was used to compare mean locomotor activity across days 7 to 10 for rats with total serum NicAb concentrations <300 µg/ml ($n = 35$) versus (300 µg/ml ($n = 14$). The proportion of animals in these subgroups exhibiting mean locomotor activity >2000 cm across days 7 to 10 were also

compared using χ^2 analysis. This cut-off (2000 cm) was used in the χ^2 analysis because it approximated the mean day 1 activity level.

Results

General

Food intake and weight gain did not differ among groups in the locomotor sensitization experiment [mean ± S.D. weight gain: control (nicotine + saline), 103 ± 19 g; vaccine, 111 ± 21 g; Nic311, 94 ± 18 g; and combined, 110 ± 24 g]. No adverse effects of antibody treatment were observed in either the Nic311 pharmacokinetic experiment or the locomotor sensitization experiment.

Nic311 Pharmacokinetic Parameter Estimates

Serum Nic311 levels in the presence or absence of nicotine are shown in Fig. 2, and pharmacokinetic parameter estimates are shown in Table 2. The Nic311 steady-state volume of distribution, clearance, and α (distribution) half-life were significantly lower in the presence of nicotine compared with its absence, but the elimination half-life was not statistically different. Three rats (depicted individually in Fig. 2) were excluded from the pharmacokinetic parameter estimation (one rat in the presence of nicotine and two rats in the absence of nicotine) because their serum nicotine levels declined considerably more rapidly after day 11, and by day 17 they were >3 S.D. below the means of their respective groups. Because rat anti-mouse antibodies were a possible cause of the more rapid decline of Nic311 levels in these outliers, ELISAs were performed on all samples collected between days 8 and 35. No anti-mouse IgG or IgM titers above background were detected in any of the rats, including the outliers.

Locomotor Sensitization

Antibody and Nicotine Levels. Cross-reactivity of serum antibodies generated by vaccination in the ELISA used to quantitate Nic311 levels was 3.2%. All reported Nic311 levels measured in rats previously vaccinated with 3′-Am-
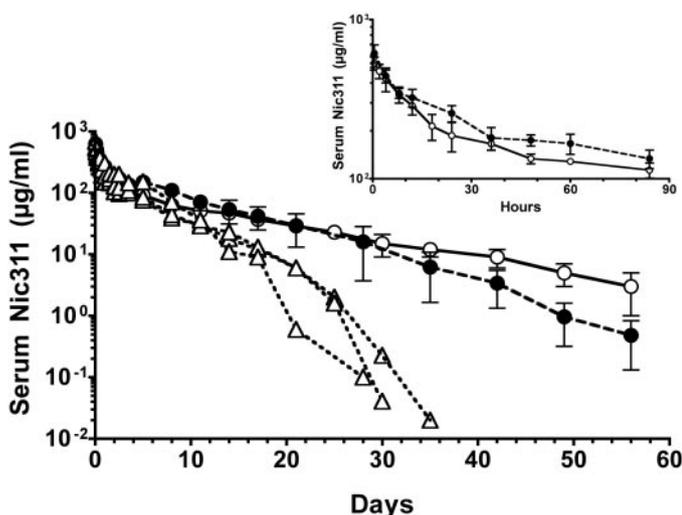

Fig. 2. Nic311 serum levels (mean ± S.D.) in the presence (●) and absence (○) of a nicotine infusion of 3.2 mg/kg/day. Inset shows levels over the initial 84 h. Individual outliers (△) were not included in calculation of the mean values. Pharmacokinetic parameter estimates are shown in Table 2.

Nic-rEPA were corrected for this cross-reactivity. Serum NicAb levels measured after the 10th nicotine dose were comparable in the groups receiving vaccination or Nic311 alone (Table 3). Total serum NicAb levels in the combined immunotherapy group were approximately double that of the two monotherapy groups ($p < 0.001$). NicAb concentrations generated by vaccination did not differ between the vaccine alone group and the combined immunotherapy group, indicating that Nic311 treatment had no effect on vaccine-elicited antibody levels. One rat receiving combined immunotherapy had an unexpectedly low serum Nic311 level reminiscent of the outliers in the pharmacokinetic experiment. The exclusion of data from this rat did not alter the results of statistical analyses, and all analyses presented include data from this rat. There was a trend toward less variability in serum NicAb levels in the combined immunotherapy group, with coefficients of variation for serum NicAb levels of 63, 46, and 35% in the vaccine alone, Nic311 alone, and combined immunotherapy groups, respectively.

Serum nicotine levels measured 40 min after the 10th (final) nicotine dose of the LMS protocol were significantly higher after vaccination ($p < 0.01$) or Nic311 alone ($p < 0.05$) compared with the nicotine control group (Fig. 3, top). Serum nicotine levels were higher after combined immunotherapy than after either vaccination or Nic311 alone ($p < 0.01$). Vaccination or Nic311 alone did not significantly reduce brain nicotine concentrations compared with controls, but brain nicotine concentrations in the combined immunotherapy group were significantly lower than in all other groups ($p < 0.01$) (Fig. 3, bottom).

Protein Binding. The percentage of nicotine bound in serum was increased and the free nicotine concentration was reduced by all antibody treatments compared with controls (Table 4), and to a significantly greater extent ($p < 0.01$) by combined immunotherapy than by either vaccination or Nic311 alone. For all groups together, there was a strong correlation between the free serum nicotine concentration and the brain nicotine concentration ($r^2 = 0.74$, $p < 0.001$) (Fig. 4).

Locomotor Activity. Figure 5 shows locomotor activity from each 30-min session throughout testing. Two-way ANOVA indicated a significant effect of group ($F = 4.35$, $p = 0.004$) and day ($F = 2.45$, $p < 0.01$), as well as a significant interaction between group and day ($F = 2.82$, $p < 0.0001$). A one-way ANOVA of overall locomotor activity on day 1 indicated no differences between groups ($F = 0.90$, $p = 0.47$) (Fig. 5).

Analysis of locomotor activity in the nicotine control group (no immunotherapy) across days indicated an overall effect of day ($F = 2.46$, $p = 0.015$). Locomotor activity on days 7 to 10 was significantly greater than on day 1 ($p < 0.05$), indicating sensitization to nicotine (mean ± S.D. increase 49 ± 77%) (Fig. 5). In contrast, locomotor activity in the saline control group declined significantly throughout testing ($F = 18.31$, $p < 0.0001$), reflecting habituation to the experimental setting (Hakan and Ksir, 1988). Locomotor activity across days did not change in the vaccine alone ($F = 0.89$, $p = 0.53$) or Nic311 alone groups ($F = 1.49$, $p = 0.16$); that is, these groups did not exhibit sensitization. The combined immunotherapy group also failed to show sensitization, and in addition, locomotor activity declined significantly across sessions

TABLE 2

Nic311 pharmacokinetic parameters with and without concurrent nicotine infusion (3.2 mg/kg/day)

Group ($n = 5$/Group)	Cl	$V_{d,ss}$	$\alpha\ t_{1/2}$	$\beta\ t_{1/2}$	AUC
	$ml/h/kg$	ml/kg	h	$days$	$mg \cdot h/ml$
Nic311	0.65 ± 0.04	163 ± 21	7.7 ± 1.6	8.0 ± 1.0	46.3 ± 3
Nic311 + nicotine	0.50 ± 0.06	122 ± 12	5.6 ± 0.9	7.6 ± 1.0	61.4 ± 8
p value	0.012	0.022	0.022	0.676	0.012

TABLE 3

Mean $\pm$ S.D. antibody concentrations among groups

	Vaccine NicAb	Nic311	Total NicAb
		$\mu g/ml$	
Vaccine	166 ± 105		166 ± 105
Nic311		172 ± 79	172 ± 79
Combined	224 ± 112	203 ± 97	420 ± 148***

*** $p < 0.001$, significantly higher than either vaccine or Nic311 alone.

Fig. 4. Relationship between free nicotine concentration in serum and the brain nicotine concentrations for rats in the locomotor sensitization protocol.

Fig. 5. Effects of treatments on locomotor sensitization. Total horizontal distance traveled (mean $\pm$ S.E.) was assessed for 30 min immediately after each daily dose of 0.3 mg/kg nicotine or saline s.c. Only the nicotine group exhibited a progressive increase in locomotor activity over the course of the experiment (sensitization). The combined immunotherapy group and the saline group showed reduced locomotor activity over the course of testing (*, $p < 0.05$ day 10 compared with day 1), and they also had significantly reduced locomotor activity compared with the vaccine alone group on days 7 to 10 (#, $p < 0.05$).
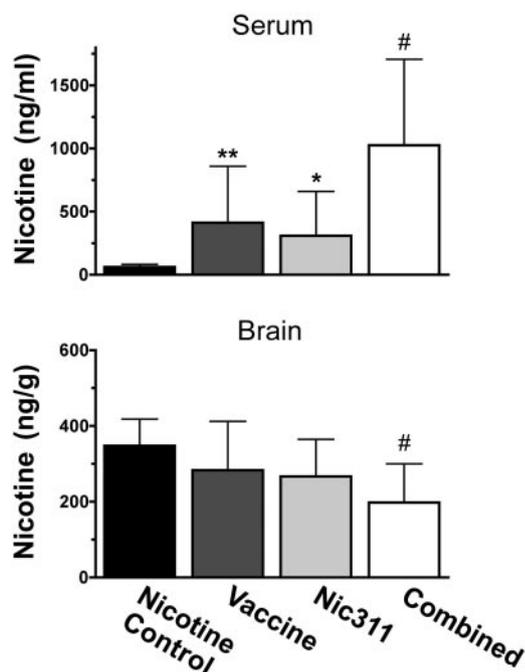

Fig. 3. Serum and brain nicotine levels (mean $\pm$ S.D.) in the locomotor sensitization experiment. Ten daily s.c. doses of 0.3 mg/kg nicotine or saline were administered, and nicotine levels were obtained 40 min after the final dose. Serum nicotine levels were significantly increased and brain nicotine levels were significantly reduced by combined immunotherapy compared with all other treatments. *, $p < 0.05$ and **, $p < 0.01$ compared with nicotine control; #, $p < 0.01$ compared with all other groups.

TABLE 4

Nicotine serum protein binding (mean $\pm$ S.D.) in serum obtained 40 min after the 10th nicotine dose in the LMS experiment

	Total Serum Nicotine	Nicotine % Bound	Unbound Nicotine
	ng/ml		ng/ml
Nicotine control	66 ± 11	8 ± 5	60 ± 10
Vaccine	452 ± 246**	84 ± 14***	47 ± 12**
Nic311	359 ± 148*	86 ± 5***	44 ± 5**
Combined	$1008 \pm 448^{\dagger\dagger}$	$95 \pm 4^{\dagger\dagger}$	$32 \pm 8^{\dagger}$

*, **, ***, $p < 0.05$, 0.01, and 0.001, respectively, compared with nicotine control.
$\dagger$ and $\dagger\dagger$, $p < 0.05$ and 0.01, respectively, compared with all other groups.

in a manner similar to the saline control group ($F = 2.804$, $p = 0.005$).

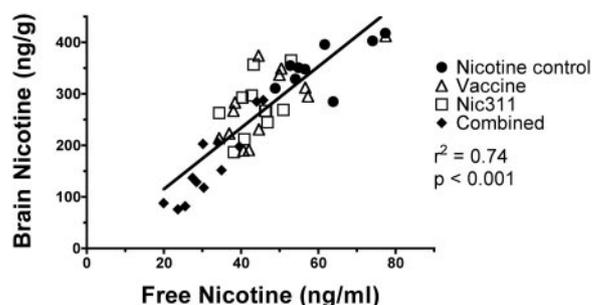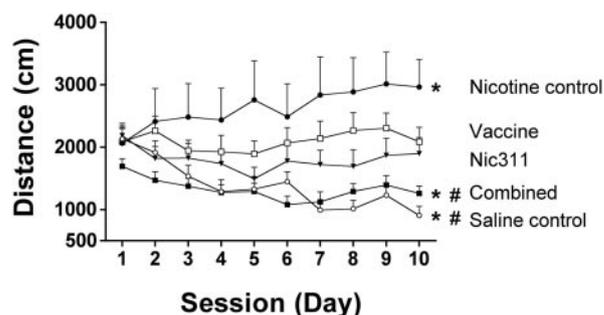Locomotor activity was compared among groups on days 7 to 10 (days that the nicotine control group exhibited sensitization). There was a significant effect of group ($F = 6.28$, $p = 0.0003$) and also a significant effect of day ($F = 3.39$, $p = 0.019$), but no significant interaction between group and day ($F = 0.57$, $p = 0.87$). Thus, overall group data across days 7 to 10 was used for post hoc comparisons. The Nic311, combined, and saline control groups had significantly lower activity levels than the nicotine control group (Fig. 5) ($p < 0.05$ for Nic311; $p < 0.01$ for saline control or combined); and there was a nonsignificant trend toward lower locomotor activity in the vaccine alone group ($p = 0.07$). The combined immunotherapy group and the saline control group had significantly lower locomotor activity compared with the vaccine alone group ($p < 0.05$).

Because the majority of activity occurs early in the locomotor sessions, data during sessions 1 and 10 (in 5-min bins) were analyzed to further clarify treatment effects (a planned comparison). Within-session analysis of locomotor activity during session 1 demonstrated a significant effect of time ($F = 125.19$, $p < 0.0001$) but no significant effect of group ($F = 0.90$, $p = 0.47$) and no interaction ($F = 1.02$, $p = 0.44$)

(Fig. 6, top). Analysis of locomotor activity during session 10 showed a significant effect of time ($F = 100.76$, $p < 0.0001$) and group ($F = 8.27$, $p < 0.0001$), and also a significant interaction ($F = 3.07$, $p < 0.0001$) (Fig. 6, bottom). The nicotine control group had significantly increased locomotor activity compared with all other groups during the initial 10 min of the session ($p < 0.001$ at 5 min; $p < 0.05$ at 10 min). The combined immunotherapy group had significantly reduced locomotor activity compared with the vaccine alone group ($p < 0.01$) and the Nic311 alone group ($p < 0.05$) during the first 5 min of the session.

Correlations. For all groups considered together, higher serum NicAb levels were associated with larger effects on nicotine pharmacokinetics by several measures. There was a significant negative correlation between the total serum NicAb concentration and free serum nicotine concentration ($r^2 = 0.36$, $p < 0.001$) and between the total serum NicAb concentration and the brain nicotine concentration ($r^2 = 0.43$, $p < 0.001$) (Fig. 7, top and middle). Higher NicAb levels were also significantly associated with greater behavioral efficacy (reduced locomotor activity) on days 7 to 10, although the relationship was weak ($r^2 = 0.11$, $p = 0.047$) (Fig. 7, bottom). In sum, rats with the highest total serum antibody concentrations had the highest total serum nicotine levels, the lowest free serum nicotine levels, the lowest brain nicotine levels, and the lowest locomotor activity scores.

The subset of rats with total serum NicAb concentrations ≥300 µg/ml (regardless of the source of the antibody) had significantly lower activity scores (1310 ± 450 versus 2080 ± 800 cm, mean ± S.D.; $p < 0.01$) and a lower proportion of rats with activity scores >2000 cm (0/14 versus 19/35; $p < 0.001$)

Fig. 7. Relationship of total serum NicAb concentration to the free serum nicotine concentration (top), brain nicotine concentration (middle), and the mean total distance traveled on days 7 to 10 (bottom).

compared with rats with total serum NicAb concentrations <300 µg/ml. The mean activity score of rats with total serum NicAb levels of ≥300 µg/ml did not differ from that of saline controls (1310 ± 450 versus 1035 ± 411 cm; $p = 0.13$).

Discussion

The main findings of this study were that 1) both vaccination alone and Nic311 alone attenuated the development of LMS to nicotine; 2) vaccination and Nic311 were equally effective in altering nicotine pharmacokinetics and attenuating LMS to nicotine when dosed to produce equivalent serum NicAb concentrations; and 3) combined immunotherapy produced higher serum NicAb concentrations, greater effects on nicotine pharmacokinetics, and greater effects on LMS than either vaccination or Nic311 alone. These data indicate that interventions that produce higher serum NicAb concentrations than vaccination alone can enhance the efficacy of immunotherapy for nicotine addiction and that the combination of vaccination and passive immunization is a feasible strategy for augmenting serum NicAb concentrations.

The effects of vaccination and of passive immunization on nicotine pharmacokinetics in rats are qualitatively similar (Hieda et al., 1999; Carrera et al., 2004; Keyler et al.,
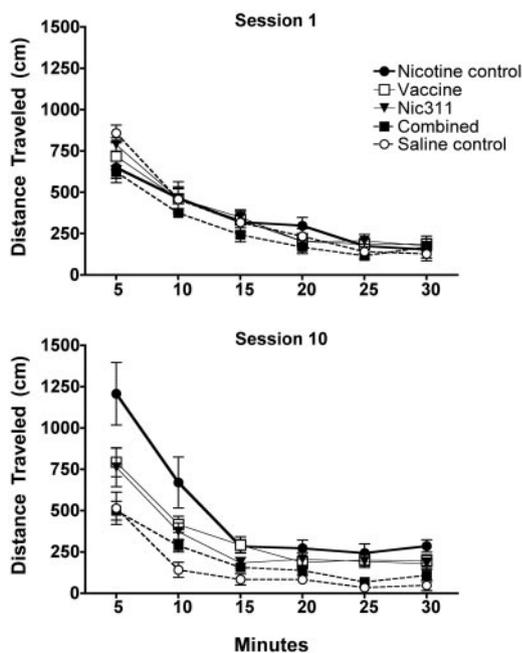

Fig. 6. Top, locomotor activity (mean ± S.E.) in 5-min blocks during the first session of the locomotor sensitization experiment. Locomotor activity did not differ among groups at any time. Bottom, locomotor activity during the 10th (final) session. The nicotine control group showed significantly greater activity at 5 min ($p < 0.001$) and 10 min ($p < 0.05$) compared with all other groups. The combined immunotherapy group had significantly reduced activity compared with rats treated with vaccine alone ($p < 0.01$) or Nic311 alone ($p < 0.05$) during the first 5 min of the session.
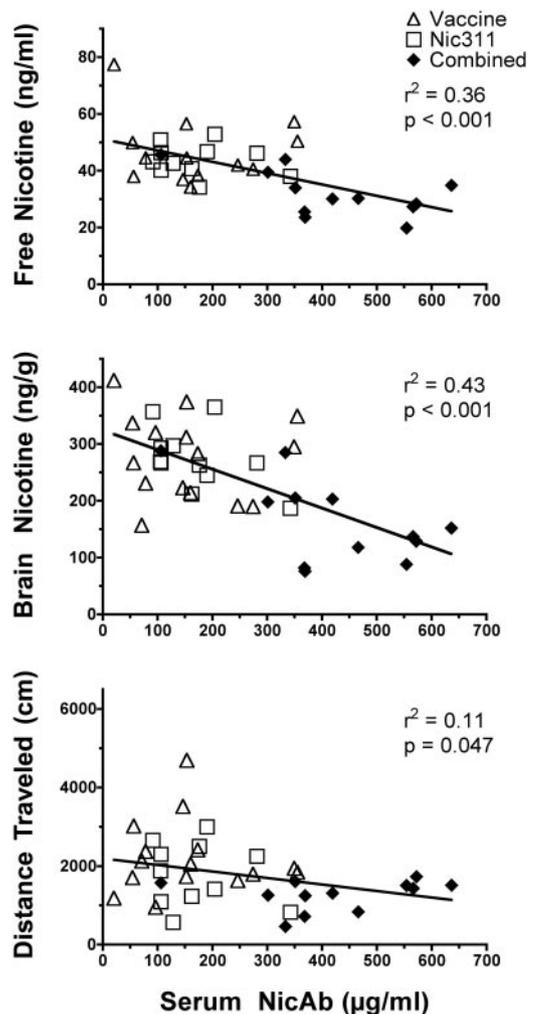
2005a,b; Pentel et al., 2006), but they have not previously been directly compared. A comparison of vaccination using 3′-AmNic-rEPA and passive immunization using Nic311 was feasible because the affinity of NicAbs elicited by vaccination is similar to that of Nic311, and the dosing regimen of Nic311 used in the LMS experiment produced serum NicAb levels equal to those generated by vaccination. The effects of these treatments on nicotine protein binding in serum and nicotine distribution to brain were comparable. Vaccination alone and Nic311 alone also both attenuated LMS to nicotine to comparable extents. Previous studies have reported attenuation of acute nicotine-induced locomotor activity (as distinct from sensitization) after passive immunization with a polyclonal anti-nicotine antiserum (Pentel et al., 2000), and vaccination or passive immunization with a monoclonal antibody (Carrera et al., 2004). The current study extends these data to LMS, a behavior mediated by pathways that overlap substantially with those mediating drug reinforcement and that is relevant to addiction treatment (DiFranza and Wellman, 2007). The data show that both vaccination alone and passive immunization alone are effective in attenuating LMS to nicotine, and they suggest that the pharmacokinetic and behavioral effects of immunotherapy depend on the total antibody concentration rather than their source (endogenously produced after vaccination or passively administered).

Combination immunotherapy (vaccination + Nic311) generated total serum NicAb levels approximately twice that of either single immunotherapy, and it was more effective than the single immunotherapies in reducing LMS to nicotine. Although all immunotherapies reduced LMS compared with the nicotine control group on days 7 to 10 of testing, only the combined treatment group (and the saline-control group that received no nicotine) showed a decrease in activity over the course of the protocol. A decrease in locomotor activity in controls has been reported by others, and the decrease is attributed to habituation to the testing procedure (Hakan and Ksir, 1988). The effects of combination immunotherapy on LMS as measured by total activity during 30 min of testing were greater than those of the vaccine group. Combination immunotherapy by this measure was not more effective than Nic311 alone. However, the attenuation of LMS by combination immunotherapy during the first 5 min of the session, during which most activity occurred, was greater than that of either vaccination or Nic311 alone. In support of the LMS data, combination immunotherapy produced greater effects on nicotine pharmacokinetics than the individual treatments alone. In addition, there were significant correlations between the total serum NicAb concentration (for all groups analyzed together) and the free nicotine concentration in serum, the brain nicotine concentration, and LMS to nicotine. Taken together, these data provide strong support for the hypothesis that increasing NicAb concentrations above those that are achievable with vaccination alone can enhance the efficacy of immunotherapy.

One prior study of immunization against nicotine in rats found no relationship between serum NicAb concentrations generated by vaccination and reductions in nicotine-self administration. However, group sizes were small, and they may not have been adequately powered to detect this relationship (LeSage et al., 2006a). Another study found that the attenuation of nicotine-induced locomotor activity by a nicotine-specific monoclonal antibody was related to antibody dose

(Carrera et al., 2004). Studies of immunotherapies for other addictive drugs have reported similar relationships between antibody doses or serum concentrations and methamphetamine-induced locomotor activity, heart rate, blood pressure, and self-administration (McMillan et al., 2004; Gentry et al., 2006), phencyclidine-induced locomotor activity (Valentine et al., 1994), and cocaine seeking and self-administration (Carrera et al., 2000; Kantak et al., 2000, 2001). Thus, most animal studies are consistent in indicating that higher serum antibody concentrations result in a greater attenuation of drug-induced behavior across a variety of drugs and immunotherapies. In two phase II clinical trials of nicotine vaccines, increased smoking cessation rates were observed only in the one third of subjects who had the highest serum NicAb titers (Hatsukami et al., 2005; Maurer and Bachmann, 2007; C. Bunce and A. Fattom, personal communication). These data support the need to achieve uniformly high serum antibody concentrations to maximize the efficacy of immunotherapy to treating drug addiction.

A previous report examined the combination of vaccination and passive immunization with a monoclonal antibody against cocaine in rats and noted that the combination was no more effective than either treatment alone in attenuating cocaine self-administration (Carrera et al., 2000). However, the study was not specifically designed to compare combination immunotherapy with vaccination or passive immunization alone, and serum antibody levels from vaccination may have been low at the time of comparison.

A limitation of this study is that the locomotor sensitization experiment did not include an untreated control group to control for effects of nonspecific antibodies: the saline group received both control vaccine and control IgG. Nonetheless, the present study provided appropriate control conditions for detecting effects of nicotine-specific antibodies. It is unlikely that nonspecific effects of either vaccination or Nic311 on locomotor activity influenced the current findings because antibodies generated from vaccination or Nic311 are highly specific for nicotine, and they do not bind endogenous neurotransmitters or the major nicotine metabolites (Pentel et al., 2000, 2006). Immunization against nicotine also produces effects that are behaviorally specific, because vaccination or passive immunization with a nicotine-specific antiserum has no effect on cocaine self-administration, cocaine-induced locomotor activity, or operant food-maintained behavior (LeSage et al., 2006b).

Although this study was not designed to establish a threshold NicAb level for efficacy, all but one of the rats in the combined immunotherapy group had serum NicAb levels of ≥300 μg/ml. Rats with serum NicAb levels above this arbitrary value exhibited levels of locomotor activity significantly lower than those with NicAb concentrations <300 μg/ml and similar to that of the saline controls. Rats with NicAb levels ≥300 μg/ml also had a significantly lower proportion of animals with activity scores >2000 cm (the approximate mean day 1 baseline) during days 7 to 10 than those with lower NicAb levels (0/12 versus 19/35). These findings suggest that a serum NicAb concentration of ≥300 μg/ml provided reliable protection against the psychoactive effects of nicotine in this protocol and that a target concentration strategy might be feasible as a rational clinical approach to immunotherapy. A threshold concentration of 300 μg/ml as discussed above is 10 times the mean serum NicAb levels reported in clinical trials,

and it is not intended as a specific target for human treatment; the LMS protocol differs in many ways from smoking and uses nicotine doses more than 10 times higher than the nicotine dose absorbed from a cigarette (Benowitz, 1996). Nonetheless, these data provide a basis for further exploration of a target antibody concentration strategy.

A target antibody concentration strategy for treating nicotine addiction would be challenging to implement clinically using currently available nicotine vaccines, owing to the large variability in serum NicAb concentrations they produce. Passive immunization alone is an alternative strategy and has been effective in blocking the effects of nicotine (Malin et al., 2001; Carrera et al., 2004; Keyler et al., 2005b), 1-(1-phenylcyclohexyl)piperidine (phencyclidine) (Valentine and Owens, 1996; Proksch et al., 2000; Hardin et al., 2002), methamphetamine (McMillan et al., 2004; Gentry et al., 2006; Pitas et al., 2006), and cocaine (Kantak et al., 2000; Norman et al., 2007) in rats, but the required doses may be quite high. The current study suggests that the combination of vaccination and passive immunization may offer some advantages. Although this study used a fixed Nic311 dose, this could be adjusted so that vaccinated rats receive only as much Nic311 as is needed to achieve a target serum NicAb concentration. The advantage of using combination immunotherapy rather than Nic311 alone lies in exploiting the ability of passive immunization to rapidly produce uniformly effective serum NicAb concentrations while minimizing the dose, the cost, and the number of subjects requiring supplemental Nic311 after vaccination.

The use of combined immunotherapy could also serve to compensate for the substantial individual variability in serum NicAb levels produced by vaccination. The coefficient of variation of the serum NicAb concentrations for the combined immunotherapy group was lower than for either of the single treatments. This suggests that the factors leading to variability in levels in the vaccine alone and Nic311 alone groups (presumably immunologic versus pharmacokinetic) are independent so that combining these approaches reduces overall variability.

Although the purpose of characterizing Nic311 pharmacokinetics in the current study was simply to guide the selection of an appropriate Nic311 dosing regimen in the LMS experiment, several interesting observations were made. The terminal half-life of Nic311 was similar to previously reported values for mouse IgG in rat (Bazin-Redureau et al., 1997), and it was not affected by the presence or absence of nicotine, but the volume of distribution, clearance, and α distribution half-life of Nic311 were modestly reduced in the presence of nicotine. However, group sizes were small, and this finding requires confirmation. An additional finding in the pharmacokinetic parameters experiment was the very rapid decline of serum Nic311 levels in several rats compared with the group mean. Rapid NicAb elimination did not seem to be due to the presence of nicotine, because two of these rats received concurrent nicotine but one did not, nor did it seem to be due to the presence of rat anti-mouse antibodies. One rat receiving combination immunotherapy in the LMS experiment also showed markedly reduced Nic311 serum levels compared with the group mean, perhaps reflecting the same process. Although excluding the data of this rat did not change the outcome of any of the statistical analyses in the LMS experiment, this observation nonetheless illustrates the value of confirming serum NicAb levels after the administration of heterologous monoclonal antibodies, for protocols lasting more than a few days.

In summary, combining vaccination with passive immunization improved the efficacy of immunotherapy for altering nicotine pharmacokinetics and attenuating nicotine-induced LMS. These data provide a basis for further study of combination immunotherapy as a means of more reliably obtaining uniformly high serum NicAb concentrations.

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