Chronic Self-Administration of Nicotine in Rats Impairs T Cell Responsiveness

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ABSTRACT

Chronic exposure of rodents to nicotine via subcutaneously or intracerebroventricularly implanted miniosmotic pumps affects T cell function. However, this method of continuous nicotine administration does not replicate the self-motivated administration of nicotine in human smokers. To determine whether nicotine impairs the immune system under conditions pertinent to human smokers, we investigated the T cell responsiveness of male Lewis rats self-administering (SA) nicotine (0.03 mg/kg of body weight per injection) 40 to 50 times/day for 5 weeks, using a model of virtually unlimited access to nicotine. Compared with sham control animals, the concanavalin A-induced proliferation of spleen cells from SA rats was significantly decreased. Moreover, the ability of spleen cells to mobilize intracellular Ca²⁺ after ligation of the T cell antigen receptor (TCR) with an anti-αβ TCR antibody was significantly less in SA than in control rats. In addition, inositol 1,4,5-trisphosphate (IP₃)-sensitive intracellular Ca²⁺ stores were markedly depleted in spleen cells from SA animals. These results suggest that chronic nicotine self-administration suppresses T cell responsiveness, and this suppression may result from an impaired TCR-mediated signaling that stems from the depletion of IP₃-sensitive intracellular Ca²⁺ stores.

Cigarette smoking is a major health risk factor and significantly increases the incidence of several diseases (reviewed in Sopori et al., 1994). It is hypothesized that this increased disease susceptibility reflects cigarette smoke-induced changes in the immune system (Holt and Keast, 1977). Chronic exposure to cigarette smoke suppresses a wide range of immunological parameters in human and animal models (Sopori et al., 1994), and this immunosuppression is associated with the particulate phase of cigarette smoke (Sopori et al., 1993). Nicotine is the major neuroactive chemical in cigarette smoke, and previous data from this and other laboratories suggest that nicotine suppresses immune and inflammatory responses (reviewed in Sopori, 1998). Chronic s.c. or i.c.v. exposure of rats to nicotine affects T cell mitogenesis and the ability of T cells to migrate from the G₀/G₁ into the S phase of the cell cycle (Geng et al., 1996, Singh et al., 2000). Ligation of the T cell antigen receptor (TCR) by anti-TCR antibodies is an in vitro model for an antigen-induced T cell activation that stimulates protein tyrosine kinases, leading to activation of phospholipase C-γ₁, production of inositol 1,4,5-trisphosphate (IP₃) (Nishibe et al., 1990, Robey and Allison, 1995), and the mobilization of intracellular Ca²⁺ due to IP₃. Effects of nicotine on T cell proliferation are associated with impaired TCR-mediated signaling in T cells, including inhibition of the ability to raise intracellular concentration of ionized Ca²⁺ ([Ca²⁺]₀) (Geng et al., 1995, 1996). Chronic round-the-clock exposure of rats to nicotine via miniosmotic pumps inhibits the [Ca²⁺]₀ response in T cells (Geng et al., 1996); however, it is difficult to assume that similar changes in T cell function will occur in human smokers, who self-administer nicotine intermittently while awake. Therefore, the present study examined the T cell function of rats that self-administered the drug with virtually unlimited access.

Materials and Methods

Animals. Male Lewis rats were purchased from Harlan (Indianapolis, IN), and food and water were available ad libitum.

Nicotine Self-Administration. Self-administration was performed according to a previously published protocol (Valentine et al., 1997; Fu et al., 2001). Briefly, 7 days after acclimatization to a reverse light cycle and handling, rats weighing 250 to 350 g were anesthetized with an intramuscular injection of xylazine-ketamine (13.87 mg/kg of body weight) (Parke-Davis, Morris Plains, NJ), and food and water were available ad libitum.

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immediately placed into operant chambers located within individual sound- and light-attenuating environmental enclosures. The jugular line, exteriorized through a polyethylene button placed between the shoulder blades, was protected by a metal spring attached to the button and connected to a dual-channel swivel outside the environmental enclosure. Rats were allowed to recover for 3 days, during which time they received progressively higher injections of heparinized saline (hourly injections of 50 µl containing 100–200 U/ml) and a daily injection of 100 µl of the antibiotic enrofloxacin (Baytril; Bayer Corporation, Shawnee Mission, KS). Following recovery, rats were randomly assigned to a self-administering (SA) group (jugular lines filled with nicotine (0.03 mg/kg of body weight in 200 U/ml heparinized saline)) or a control group (heparinized saline). Rats were kept on a 12-h light/dark cycle (12:30 AM to 12:30 PM).

The interior operant chamber has two levers with green cue lights signaling the availability of nicotine or saline. The levers were randomly assigned as active (programmed delivery of 50 µl of nicotine or saline within 0.81 s when pressed by the rat) or inert (unproductive pressing). Injections were followed by a 7-s time-out (cue lights out and nicotine/saline unavailable). During the adaptation period (approximately 1 week), the frequency of nicotine self-administration varied considerably among the individual animals. However, after the first week (maintenance phase), a majority of the animals self-administered nicotine ( = 0.03 mg/kg of body weight) 40 to 50 times/day for 5 weeks; these animals were selected for the study.

**Determination of Cotinine Levels in Blood Plasma.** Blood samples were taken from animals after 20 days in the maintenance phase of nicotine self-administration, placed immediately into EDTA-containing tubes on ice, and spun at 4°C to collect the plasma. One milliliter of a plasma sample was extracted with 1 ml of sodium tetraborate (20 g/l), 3 ml of 50:50 dichloromethane/dichloroethane (Sigma-Aldrich, St. Louis, MO), and 100 ng of deuterated cotinine (Cerilliant Corporation, Austin, TX). The sample extract (lower layer of centrifuged solution) was decanted in a scintillation vial, evaporated under a gentle stream of nitrogen, and reconstituted in 1 ml of analytical-grade methanol (Fisher Scientific, Fair Lawn, NJ). Analysis was conducted by high-pressure liquid chromatography (Shimadzu SCL-10A; Shimadzu Corporation, Kyoto, Japan) coupled to a triple quadrupole mass spectrometer (API 365; Applied Biosystems, Foster City, CA). Mass spectrometry analysis was conducted in the positive ionization mode using a turbo ion spray ionization source. Parent/daughter ions for cotinine/deuterated cotinine were monitored at 177.80 and 180.80. Analyte concentrations were determined as the ratio of the compound area to the area counts of the spiked deuterated analog. Differences in the response between deuterated compounds and target analytes were compensated by creating calibration curves that spanned the range of the sample concentrations.

**Preparation of Spleen Cells.** Spleen cell suspensions were prepared as described elsewhere (Geng et al., 1995). Briefly, spleens were passed through stainless steel mesh, and red blood cells were lysed by treatment with NH4Cl solution. After washing three times with phosphate-buffered saline (PBS), cells were suspended in complete medium (RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, 50 mM 2-mercaptoethanol, and 10 µg/ml gentamicin).

**Assay for Concanavalin A (Con A)-Induced Proliferation.** Response of spleen cells to the T cell mitogen Con A (Sigma-Aldrich) was assayed as described previously (Geng et al., 1995). Briefly, 5 × 10⁶ cells were cultured in 0.2 ml of complete medium in microtiter wells in the presence and absence of indicated concentrations of Con A. The cultures were incubated at 37°C in the presence of 5% CO₂ and harvested after 3 days. Cell proliferation was assessed by pulsing the culture wells with 0.5 µCi of [3H]thymidine 12 h before harvesting.

**Assay for [Ca²⁺], and IP₃-Sensitive Ca²⁺ Stores.** [Ca²⁺], was determined by spectrofluorometry as described previously (Razani-Boroujerdi et al., 1994). Briefly, spleen cells were loaded with acetoxymethyl ester of indo 1 (Sigma-Aldrich), and changes in [Ca²⁺] were recorded by a Deltascan fluorometer (Photon Technology International, South Brunswick, NJ). The baseline [Ca²⁺], of indo 1-loaded cells was recorded before the addition of 2.5 µg/ml anti-rat αβ-TCR monoclonal antibody (mAb; BD PharMingen, San Diego, CA) and the second antibody (Ab; 2.5 µg/ml goat anti-mouse IgG; Sigma-Aldrich). To determine the concentration of Ca²⁺ within the IP₃-sensitive Ca²⁺ stores, indo 1-labeled cells were suspended in Ca²⁺-, Mg²⁺-free PBS containing 100 µM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid and treated with 1 mM thapsigargin (Sigma-Aldrich) (Kalra et al., 2000). The excitation wavelength for indo 1 is 355 nm, and emission was measured at 410 and 485 nm. After subtracting the background, [Ca²⁺], was calculated as described elsewhere (Razani-Boroujerdi et al., 1994).

**Results**

**Self-Administration of Nicotine Suppresses T Cell Mitogenesis.** Chronic exposure to mainstream cigarette smoke and nicotine suppresses the ability of T cells to proliferate in response to antigens and mitogens (Sopori and Kozak, 1998). To determine whether chronic self-administration of nicotine affected T cell proliferation, spleen cells from control and nicotine SA rats were treated with Con A. Results (Fig. 1) show that nicotine self-administration significantly decreased the ability of T cells to proliferate at all the concentrations of Con A tested in the experiment. Thus, self-administration of nicotine affects the proliferative response of T cells.

**Blood Cotinine Levels.** The half-life of nicotine in rodents is extremely short (Mactutus, 1989). To ascertain that the self-administration did not produce unusually high exposures to nicotine, plasma concentrations of cotinine, a relatively stable (half-life > 10 h in the blood) metabolite of nicotine, were analyzed by high-pressure liquid chromatography. Figure 2 shows that cotinine levels of nicotine SA plasma were significantly higher than those of control plasma (9.0 ± 1.5 ng/ml and 0.6 ± 0.03 ng/ml; p = 0.016). However, these concentrations are several-fold lower than

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![Fig. 1](https://example.com/f1.png)  
**Fig. 1.** Con A proliferative response. Spleen cells from control (CON) and SA rats were treated with indicated concentrations of Con A for 3 days. Cell proliferation was determined as described under Materials and Methods. Results are representative of two separate experiments. *, values are statistically significant from CON (p < 0.05).
observed in the nicotine SA animals), suggesting that self-administration of nicotine may affect the antigen-mediated activation of T cells through inhibition of the TCR signaling pathway at step(s) proximal to Ca$^{2+}$ mobilization.

**Nicotine Self-Administration Depletes IP$_3$-Sensitive Ca$^{2+}$ Stores in T Cells.** Increases in cell [Ca$^{2+}$]$_i$ are dependent on the intracellular levels of IP$_3$. Increased IP$_3$ concentrations trigger release of Ca$^{2+}$ from IP$_3$-sensitive Ca$^{2+}$ stores in the sarcoplasmic/endoplasmic reticulum, which stimulates Ca$^{2+}$ influx through a capacitative action (Haverstick and Gray, 1993). The inability of T cells from SA animals to raise [Ca$^{2+}$]$_i$ in response to TCR activation may result from inadequate production of IP$_3$ and/or inadequate levels of Ca$^{2+}$ in the IP$_3$-sensitive Ca$^{2+}$ stores. Results presented in Fig. 4 suggest that when spleen cells are treated with thapsigargin, an agent that primarily releases Ca$^{2+}$ from IP$_3$-sensitive Ca$^{2+}$ stores (Takemura et al., 1989; Gouy et al., 1990), in a Ca$^{2+}$-free medium (to discount the effects of Ca$^{2+}$ influx on [Ca$^{2+}$]$_i$), the increase in [Ca$^{2+}$]$_i$ was substantially lower (30–40%) in nicotine SA than in control animals. Thus, chronic self-administration of nicotine depletes IP$_3$-sensitive Ca$^{2+}$ stores.

**Discussion**

The lungs of a two-pack smoker receive 40 to 80 mg of nicotine per day (American Medical Association, 1986), which translates into 0.6 to 1.2 mg of nicotine per kg of body weight per day. Daily s.c. exposure of rats to these concentrations of nicotine for 3 to 4 weeks via constant release miniosmotic pumps affected the antigen-mediated signaling in T cells (Geng et al., 1995, 1996), indicating that chronic administration of nicotine impairs T cell function. However, when the same concentrations of nicotine were administered through two daily intraperitoneal injections for 3 weeks, none of the immunological parameters was affected (Geng et al., 1995). Therefore, the manner in which nicotine is administered is important in determining its effects on the immune system. Depending on the nature of the stimulus and the

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![Fig. 2. Plasma cotinine levels in control (CON) and SA animals. High-pressure liquid chromatography for cotinine was performed as described under Materials and Methods. Values represent means of five replicates. * values are statistically significant from CON (p < 0.05).](image2)

![Fig. 3. Effect of SA on the TCR-induced rise in [Ca$^{2+}$]. Spleen cells were loaded with Indo 1 and incubated with mouse anti-rat, anti-TCR mAb followed by goat anti-mouse polyclonal Ab (second Ab). [Ca$^{2+}$]$_i$ was calculated as described under Materials and Methods. Results are representative of four separate control (CON)/SA animal combinations.](image3)

![Fig. 4. Measurement of IP$_3$-sensitive Ca$^{2+}$ stores. Indo 1-labeled spleen cells of SA and control (CON) were suspended in Ca$^{2+}$-free PBS containing 100 μM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N’-tetraacetic acid and treated with 1 mM thapsigargin. [Ca$^{2+}$]$_i$ was calculated as described under Materials and Methods. Results are representative of four separate control (CON)/SA animal combinations.](image4)
differentiation state of the T cells, signaling through TCR can lead to profound biological responses, including activation, tolerance, and/or differentiation (Sloan-Lancaster and Allen, 1996). Therefore, it is essential to ascertain whether the immunological changes associated with chronic, constant nicotine treatment reflect the effects on the immune system that are specific to this method of nicotine administration (i.e., constant exposure). Nicotine exposure associated with cigarette smoking in humans is a chronic daily activity and restricted to conscious hours. In the past, it has been difficult to replicate this method of nicotine exposure in animals. However, Valentine et al. (1997) demonstrated the feasibility of chronic nicotine self-administration in rats, and in the present study that protocol was used to expose rats for 5 weeks to nicotine. These animals self-administered nicotine 40 to 50 times/day, receiving 0.03 mg/kg of body weight each time (i.e., approximately 1.2 mg of nicotine/kg of body weight per day). This dosage is similar to the concentration that caused functional defects in T cells of rats after 3 to 4 weeks of exposure via miniosmotic pumps (Geng et al., 1995, 1996). However, compared with the cotinine levels (>100 ng/ml) obtained through constant nicotine exposure via miniosmotic pumps, self-administration produced much lower plasma cotinine levels (9 ng/ml), indicating that animals were not exposed to unusually high levels of nicotine. Despite lower plasma cotinine levels, nicotine self-administration significantly suppressed the Con A-induced T cell mitogenesis. Because the proliferative response to Con A is an indicator of cell-mediated immunity, these results suggest that, as in human smokers (Sopori et al., 1994), self-administration of nicotine suppresses the cell-mediated immune response. It was possible that changes in the proliferative response reflected differences in the proportion of splenic T cells between control and nicotine-treated animals. However, in a previous study (Geng et al., 1995), chronic nicotine treatment (3–4 weeks by s.c. miniosmotic pumps) did not affect the number or the subset distribution of lymphocytes. Therefore, it is likely that nicotine self-administration affects the activity but not the number of T cells. It is possible that nicotine affects the responses of immune cell types other than T cells; however, in this study we focused on the effects of nicotine self-administration on T cell function.

The ability of T cells to increase [Ca\(^{2+}\)]\(_i\), is an early step in antigen-mediated T cell activation and proliferation (Weiss and Littman, 1994). Nicotine self-administration impairs the ability of T cells to mobilize ionized Ca\(^{2+}\), which is critical for the progression of T cells from the G0/G1 to the S phase of the cell cycle (Clapham, 1995; Takuwa et al., 1995). Defects in T cell signaling in response to Con A are also observed in T cells from human cigarette smokers (Suzuki et al., 1999) and rats given chronic cigarette smoke (Kalra et al., 2000). Our results suggest that nicotine self-administration may affect step(s) in the T cell signaling cascade that is/is proximal to the Ca\(^{2+}\) response. Preceding the antigen-stimulated rise in [Ca\(^{2+}\)]\(_i\), T cells increase the levels of IP\(_3\) through activation of phospholipase C-γ1 (Weiss and Littman, 1994). IP\(_3\) binds to and releases Ca\(^{2+}\) from IP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores (Clapham, 1995). The released Ca\(^{2+}\) acts in a capacitative manner to trigger Ca\(^{2+}\) influx (Haverstick and Gray, 1993), leading to increased [Ca\(^{2+}\)]\(_i\). To determine the status of the IP\(_3\)-sensitive Ca\(^{2+}\) stores, cells were treated in a Ca\(^{2+}\)-free medium with thapsigargin, which inhibits the endoplasmic reticulum Ca\(^{2+}\)-ATPases (Thastrup et al., 1990), resulting primarily in uncompensated depletion of the IP\(_3\)-sensitive Ca\(^{2+}\) stores (Jackson et al., 1988). These Ca\(^{2+}\) stores are critically important in the communication between the cytoplasm and the nucleus (Greber and Gerace, 1995; Perez-Terzic et al., 1997). Results presented herein suggest that self-administration of nicotine significantly depletes these pools, and this depletion could affect the ability of T cells to mobilize ionized Ca\(^{2+}\) in response to TCR activation.

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References


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