Differential Effects of Passive Immunization with Nicotine-Specific Antibodies on the Acute and Chronic Distribution of Nicotine to Brain in Rats

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ABSTRACT
Vaccination against nicotine blocks or attenuates nicotine-related behaviors relevant to addiction in rats. Passive immunization with nicotine-specific antibodies is an alternative to vaccination with the potential advantages of allowing control of antibody dose and affinity. In the current study, the effects of two antibodies on the distribution of nicotine to brain were evaluated during chronic nicotine administration in rats; the monoclonal antibody Nic311 ($K_d = 60$ nM) and nicotine-specific antiserum ($K_d = 1.6$ nM). Nicotine was administered via repeated i.v. bolus doses over 2 days and antibody was administered during the first day. Neither antibody appreciably reduced the chronic accumulation of nicotine in brain, despite high protein binding of nicotine in serum (98.9%) and a 73% reduction in the unbound serum nicotine concentration with the highest Nic311 dose. However, both antibodies substantially reduced the early distribution of nicotine to brain 5 min after a dose. The higher affinity antibody was no more effective than Nic311. The highest Nic311 dose produced serum antibody levels 10 times higher than those reported with vaccination. The efficacy of Nic311 was dose-related, with the highest dose producing a 76% decrease in the early distribution of nicotine to brain. These findings, along with previous data, suggest that the primary effect of passive immunization is to slow, rather than prevent, the distribution of nicotine to brain. In the setting of chronic nicotine dosing, antibodies with a moderate affinity for nicotine produced substantial effects on the early distribution of nicotine to brain and were as effective as higher affinity antibodies.

Nicotine vaccines are of interest as potential treatments for tobacco dependence. Vaccination (active immunization) of rats against nicotine elicits nicotine-specific antibodies that bind nicotine, reduce nicotine distribution to brain (Keyler et al., 1999; Cerny et al., 2002; Satoskar et al., 2003), and attenuate a variety of behaviors relevant to addiction, including nicotine self-administration (Malin et al., 2001; Lindblom et al., 2002; Malin et al., 2002; LeSage et al., 2006). Three nicotine vaccines in phase II or III clinical trials have proven safe, and two of these vaccines, in secondary analyses, demonstrated some efficacy for smoking cessation (Hatsukami et al., 2005; M. Bachmann, personal communication). Vaccination is an attractive treatment because of its specificity, safety, and long-lasting effect, which could improve treatment compliance (Voci and Chiang, 2001; Pentel and Keyler, 2004). However, the amount of antibody that can be generated by vaccination is limited to approximately 1 to 2% of total immunoglobulin and shows considerable individual variability, which might limit its efficacy (Satoskar et al., 2003; Hatsukami et al., 2005). In addition, the affinity and specificity of antibody generated may vary among individuals.

An alternative to vaccination that could address these limitations is passive immunization via the transfer of nicotine-specific antibody (Kosten and Owens, 2005). Rabbit antiserum containing polyclonal nicotine-specific antibodies administered to rats has effects similar to those of vaccination, and the potential advantage of providing even greater efficacy if a sufficient dose is used (Pentel et al., 2000). Use of a monoclonal antibody rather than antiserum could further allow control of antibody affinity and specificity and provide a therapeutic agent with reproducible properties. One such monoclonal antibody has been shown to block nicotine-induced locomotor activity despite having only a moderately low affinity for nicotine.

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ABBREVIATIONS: Nic311, nicotine-specific monoclonal antibody; Nic-IgG, nicotine-specific rabbit antiserum; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.
low $K_d$ value for nicotine of 200 nM (Carrera et al., 2004). However, the influence of antibody affinity and dose on efficacy is not well established. We recently studied a series of nicotine-specific monoclonal antibodies with regard to their ability to block the distribution to brain of a single nicotine dose in rats (Keyler et al., 2005b). Efficacy was strongly related to affinity, with the highest affinity monoclonal dose in rats (Keyler et al., 2005b). Efficacy was strongly related to affinity, with the highest affinity monoclonal (Nic311; $K_d$ of 60 nM) more effective than others with $K_d$ values of 70 or 250 nM. Rabbit antiserum with a $K_d$ of 1.6 nM was more effective than any of the monoclonals. However, the efficacy of Nic311 was dose-related, so it was possible to achieve equivalent or greater efficacy with Nic311 than with rabbit antiserum by providing a sufficient Nic311 dose.

These data support the potential use of monoclonal antibodies to treat nicotine dependence, but antibody effects were examined using only a single dose of nicotine. In the current study, we investigated the relationship between antibody dose, affinity, and efficacy, and used a well characterized regimen of repeated nicotine doses that approximates the daily nicotine intake of a smoker (LeSage et al., 2003). The main hypothesis was that Nic311 would reduce nicotine distribution to brain in a dose-related manner but that it would be less effective than Nic-IgG. Effects on the chronic accumulation of nicotine in brain were measured as well as effects on the early distribution of nicotine to brain after a single dose administered concurrently with the chronic nicotine. Nic311 was used in this study because its effects on single dose nicotine distribution have been previously characterized, and it has the highest affinity of antinicotinic monoclonal antibodies identified to date. The rabbit antiserum Nic-IgG was also used for comparison, since it has a still higher affinity for nicotine, to allow study of the effects of antibody affinity on efficacy.

**Materials and Methods**

**Antibodies.** Monoclonal and polyclonal nicotine-specific antibodies were elicited using the immunogen nicotine-3'-aminomethyl nicotine as described previously (Keyler et al., 2005b). The monoclonal antibody Nic311 is an IgG1 with a $K_d$ for nicotine of 60 nM. Nic311 was produced from hybridoma cells grown in spinner flasks in serum-free medium and purified by protein G chromatography. Nic311 concentration was measured by ELISA, was 5% of total protein. The Nic-IgG doses used in this study were expressed as the ratio of the $ED_{50}$ for inhibition of Nic311 binding for each compound tested and the $ED_{50}$ for inhibition of binding by nicotine. Antibody was diluted in phosphate-buffered saline for administration. Rabbit nicotine-specific antibody (Nic-IgG) was prepared as described previously from immune antiserum, and total IgG was purified by protein G chromatography (Pentel et al., 2000). The nicotine-specific IgG content of the resulting product, determined by ELISA, was 5% of total protein. The Nic-IgG doses used in this study are expressed as the concentration of the nicotine-specific IgG. Control antibody was human nonspecific IgG (SanDorglobulin; Sandoz, Vienna, Austria). This antibody has been shown to have negligible binding to nicotine and no effect on nicotine distribution in the rat (Keyler et al., 2005b).

**Nicotine.** (−)-Nicotine bitartrate (Sigma-Aldrich, St. Louis, MO) was diluted in phosphate-buffered saline for dosing via infusion pump. We added 3 μCi of [3H]nicotine (Sigma-Aldrich) with specific activity 87 Ci/mmol to the final unlabeled nicotine dose to allow the disposition of the final nicotine dose to be measured independently of the accumulated nicotine from previous doses (Hieda et al., 2000). Unlabeled nicotine concentrations were measured by gas chromatography with nitrogen phosphorus detection (Jacob et al., 1981). Labeled nicotine concentrations were measured by scintillation counting. All nicotine doses and concentrations are expressed as weight of the dose. Brain nicotine concentrations were corrected for brain blood content (Hieda et al., 1999).

**Protein Binding.** Equilibrium dialysis of serum was performed in 1.0-ml Teflon cells with Spectrapor 2 membranes (Spectrapor Labs, Rancho Dominguez, CA) against phosphate-buffered saline, pH 7.4, for 4 h at 37°C (Pentel and Keyler, 1988). Only unlabeled nicotine concentrations were measured because it was reasoned that the binding of [3H]nicotine present in serum could have changed during the 4 h required for dialysis and would no longer accurately reflect events taking place 5 min after the last nicotine dose.

**Protocol.** Male Holtzman Sprague-Dawley rats weighing 250 to 300 g were housed individually during the experiment with a lights on 10:00 AM under a 12:12-h light/dark cycle. Rat group size was $n = 3$ for those receiving Nic311 or Nic-IgG and $n = 5$ for controls. The small group size was dictated by the cost and availability of these antibodies. Only two doses of Nic-IgG were used (10 and 30 mg/kg) because it contains only 5% nicotine-specific IgG, and administration of larger doses would have entailed administering very large total protein loads. Rats were anesthetized with droperidol/fentanyl for placement of jugular and femoral venous catheters. Nicotine administration was started 12 h later at 10:00 PM ($t = 0$) via the jugular catheter as bolus doses of 30 μg/kg every 14 min for 16 h/day, delivered in a volume of 50 μl over 1 s by infusion pump (Fig. 1). The total daily dose was 2 mg/kg. This well characterized regimen was chosen because it provides daily nicotine doses and peak nicotine concentrations in serum similar to those of heavy smokers (LeSage et al., 2003), and the unit dose of 30 μg/kg is both well tolerated and reinforcing in rats (Corrigall and Coen, 1989). In addition, the 16 h/day schedule resembles a typical smoking pattern. Antibody was administered at $t = 12$ h. The 12-h interval before administering antibody allowed the serum nicotine concentration to approximate steady state to model antibody use in a current smoker. Antibody was administered via the femoral catheter at doses of 10 and 30 mg/kg for Nic-IgG (dose of the nicotine-specific antibody content); 10, 30, 80, and 240 mg/kg for Nic311; and 80 mg/kg for control-IgG. Antibody was infused over 4 min in a volume of 2 ml for the 240-mg/kg dose of Nic311 and the 30-mg/kg dose of Nic-IgG, and in a volume of 1 ml for all other doses. At $t = 40$ h, at the end of the second 16-h period of nicotine dosing, the infusion pump was disconnected. Fourteen minutes later, 30 μg/kg nicotine containing [3H]nicotine was administered via the femoral catheter manually over 5 s. Addition of [3H]nicotine to this final dose allowed its distribution to be measured independently of that of the accumulated previous nicotine doses. Five minutes after this dose, rats were decapitated, and trunk blood and brain were rapidly collected. Serum and brain were frozen at −20°C until analyzed.

**Data Analysis.** For stoichiometric calculations, antibody binding site doses were calculated assuming a molecular mass of 150 kDa for Nic311 or Nic-IgG, two binding sites per IgG, and a molecular mass of 162 Da for nicotine. Serum or brain nicotine concentrations were measured by gas chromatography with nitrogen phosphorus detection (Jacob et al., 1981). Labeled nicotine concentrations were measured by scintillation counting. All nicotine doses and concentrations are expressed as weight of the dose. Brain nicotine concentrations were corrected for brain blood content (Hieda et al., 1999).

![Fig. 1. Experimental protocol.](image-url)
compared across groups by one-way ANOVA with Tukey’s test to compare individual groups if the overall ANOVA was significant. Protein binding among groups was compared using one-way ANOVA with Dunnett’s test. The relationship between antibody dose and nicotine concentrations was analyzed by linear regression.

**Results**

**Antibody.** Nic311 specificity was tested against a range of compounds having either structural or functional similarities, including the principal metabolites of nicotine and the endogenous nicotinic receptor ligand acetylcholine (Table 1). None of these compounds had appreciable cross-reactivity with nicotine, with most having ED$_{50}$ values less than 1% that of nicotine. Mean serum antibody concentrations measured at the time of sacrifice were 100 ± 10, 270 ± 10, 560 ± 60, and 1600 ± 130 μg/ml for Nic311 doses of 10, 30, 80, and 240 mg/kg, and 70 ± 10 and 220 ± 40 μg/ml for Nic-IgG doses of 10 and 30 mg/kg.

**Serum Nicotine Concentrations.** The total (unlabeled) serum nicotine concentration (Fig. 2, top), representing the accumulation of the chronically administered nicotine, was increased compared with controls by treatment with all except the lowest Nic311 dose, and by both doses of Nic-IgG ($p < 0.01$). These increases were dose-related as indicated by significant differences between each of the treatment groups ($p < 0.01$). The 30-mg Nic-IgG dose produced a greater increase in serum nicotine concentration than the 30-mg Nic311 dose, but the effects of the 10-mg doses of these antibodies did not differ. The serum $[^{3}H]$nicotine concentration (Fig. 3, top), representing the final nicotine dose, was also significantly increased by all Nic311 and Nic-IgG doses compared with controls ($p < 0.01$). As with the unlabeled nicotine, the 30-ng Nic-IgG dose produced a greater increase in the serum $[^{3}H]$nicotine concentration than 30-ng Nic311 dose, but the effects of the 10-mg doses of these antibodies did not differ.

**Brain Nicotine Concentrations.** There were no differences in the brain unlabeled nicotine concentration for any of the Nic311 or Nic-IgG doses as determined by ANOVA (Fig. 2). There was a significant correlation between the serum Nic311 concentration and the brain nicotine concentration. However, the magnitude of this effect was small, with an 18% change in brain nicotine concentration over a 10-fold range of Nic311 concentrations (Fig. 4, top). In contrast to the unlabeled concentrations, brain $[^{3}H]$nicotine concentrations were reduced by all Nic311 or Nic-IgG doses except 10 mg/kg Nic-IgG (Fig. 3). The highest (240 mg/kg) Nic311 dose reduced $[^{3}H]$nicotine distribution to brain by 76%. There was no difference in brain $[^{3}H]$nicotine concentrations between the 30-mg/kg Nic311 and Nic-IgG- or the 10-mg/kg Nic311 and Nic-IgG groups. Brain $[^{3}H]$nicotine concentrations were highly correlated with the serum Nic311 concentration (Fig. 4, bottom), and the magnitude of this effect was large, with a 64% change in brain nicotine concentration over a 10-fold range of Nic311 concentrations.

**Serum Cotinine Concentrations.** There were no significant differences between serum cotinine concentration for any groups compared with controls (Fig. 5).

**Protein Binding.** The binding of unlabeled nicotine in serum was substantially increased by Nic-IgG, and by Nic311 in a dose-related manner (Table 2). The unbound nicotine concentration was reduced by all treatments.

**Stoichiometric Relationships.** The molar Nic311 or Nic-IgG doses administered to each group, and their ratios

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Cross-Reactivity$^a$</th>
</tr>
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<tbody>
<tr>
<td>Nicotine</td>
<td>100</td>
</tr>
<tr>
<td>Cotinine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Nornicotine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Nicotine-$N$-oxide</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Anatabine</td>
<td>1.9</td>
</tr>
<tr>
<td>4-Methylnitosamine-1-(3-pyridyl)-1-butanol</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cytisine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Dihydro-$\beta$-erythroidine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Mecamylamine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Choline</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Serotonin</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Dopamine</td>
<td>&lt;1</td>
</tr>
<tr>
<td>$\gamma$-Aminobutyric acid</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cocaine</td>
<td>&lt;1</td>
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</tbody>
</table>

$^a$ Determined from the ratio of the ED$_{50}$ of each compound for inhibition of Nic311 binding by ELISA compared with the ED$_{50}$ for inhibition of binding by (-)-nicotine.
compared with the final [3H]nicotine dose of 30 μg/kg are shown in Table 3. These relationships indicate that the final nicotine dose exceeded the binding capacity of the lowest antibody dose, but it represented only a fraction of the higher antibody doses.

**Discussion**

Neither Nic311 nor Nic-IgG had an appreciable effect on the chronic accumulation of nicotine in brain as measured by ANOVA. There was a significant relationship between the serum Nic311 concentration and the nicotine concentration in brain by correlation analysis, but the actual differences in brain nicotine concentrations were small. In contrast, both Nic-IgG and Nic311 had marked and dose-related effects on the early distribution of [3H]nicotine to brain 5 min after a nicotine dose, and the differences among groups were large. These findings are similar to those from rats vaccinated (actively immunized) against nicotine with this same immunogen, which, in various studies, either did not affect chronic nicotine accumulation in brain or had much less effect than on the early distribution of nicotine to brain following a single concurrently administered dose (Hieda et al., 2000; Keyler et al., 2005a). The current study extends these findings to passive immunization and shows that they occur over a wide range of antibody doses and antibody concentrations considerably higher than those achievable with vaccination. Together, these data suggest that nicotine-specific antibodies, regardless of their source, serve primarily to slow nicotine distribution to brain, blunting the early peak exposure but having less effect at later times. Although the
Slower drug delivery to brain, in general, is associated with less distribution to brain because the reinforcing and amine distribution to brain could be due to differences in the time course of antibody effects on nicotine and methamphetamine, higher total clearance than nicotine in the rat (Keyler et al., 1999, 2005b), could also contribute to changes in nicotine-related behaviors. Genetic polymorphisms with similar effects on nicotine clearance have been found to be associated with reduced smoking in humans (Malaiyandi et al., 2005).

TABLE 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Antibody Dose</th>
<th>[3H]Nicotine Dose</th>
<th>Antibody: [3H]Nicotine Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nic311, 10 mg/kg</td>
<td>0.13</td>
<td>0.19</td>
<td>0.7</td>
</tr>
<tr>
<td>30</td>
<td>0.40</td>
<td>0.19</td>
<td>2.2</td>
</tr>
<tr>
<td>80</td>
<td>1.10</td>
<td>0.19</td>
<td>5.9</td>
</tr>
<tr>
<td>240</td>
<td>3.20</td>
<td>0.19</td>
<td>16.7</td>
</tr>
<tr>
<td>Nic-IgG, 10 mg/kg</td>
<td>0.13</td>
<td>0.19</td>
<td>0.7</td>
</tr>
<tr>
<td>30</td>
<td>0.40</td>
<td>0.19</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Expressed as binding sites, assuming two binding sites per IgG.

The reported efficacy of vaccination against nicotine in attenuating nicotine self-administration in rats, a setting that involves chronic repeated nicotine dosing similar to that used in the current study, supports the potential importance of this early effect (LeSage et al., 2006). However, additional effects of immunization, including reduced nicotine clearance seen with both vaccination and with Nic311 treatment (Keyler et al., 1999, 2005b), could also contribute to changes in nicotine-related behaviors. Genetic polymorphisms with similar effects on nicotine clearance have been found to be associated with reduced smoking in humans (Malaiyandi et al., 2005).

In a previous study examining antibody effects on a single nicotine dose (without concurrent chronic nicotine), the higher affinity polyclonal Nic-IgG ($K_a$ of 1.6 nM) was considerably more effective than Nic311 ($K_a$ of 60 nM) in reducing nicotine distribution to brain 5 min after nicotine dosing; 10 mg/kg Nic-IgG was as effective as 80 mg/kg Nic311 (Keyler et al., 2005b). In the current study, the serum protein binding of nicotine was greater with Nic-IgG than at equivalent doses of Nic311, so that Nic-IgG was more highly saturated with nicotine than was Nic311. This may have left more binding sites on the Nic311 available to bind the next nicotine dose and contributed to the nearly equal efficacy of these antibodies under chronic nicotine dosing conditions. A potential confounder in this study is that Nic-IgG serum levels were lower than those resulting from the same dose of Nic311, but the difference was small and not likely to be a major contributor to the lack of a meaningful advantage of Nic-IgG over Nic311.

The substantial reduction of nicotine distribution to brain after Nic311 treatment in this study is notable because the doses of nicotine used were large compared with the binding capacity of the antibody. The daily nicotine dose of 2 mg/kg was approximately 4 times the binding capacity of the highest Nic311 dose used, and 96 times that of the lowest Nic311 dose. However, the higher antibody doses were much larger on a molar basis than each individual nicotine dose, perhaps explaining the ability of the antibody to transiently alter the distribution of each individual dose despite a high degree of antibody saturation with nicotine.

The comparable efficacy of Nic311 and Nic-IgG is important because antibodies with $K_a$ values lower than 60 nM have not been reported to date in vaccinated rats or from murine monoclonal antibodies, despite the use of seven additional nicotine immunogens distinct from that used to generate Nic311 (Hieda et al., 1997; Cerny et al., 2002; Lindblom et al., 2002; Meijler et al., 2003; Sanderson et al., 2003; Carrera et al., 2004; Maurer et al., 2005). The $K_a$ values of antibodies elicited in humans in clinical trials of nicotine vaccines have not been reported. Thus, generating higher affinity antinicotinic antibodies has proven difficult. The effects of Nic311 found in this study suggest that an antibody with a $K_a$ for nicotine of 60 nM may be sufficient to produce therapeutic effects. In support of this possibility, rats vaccinated with the same immunogen used to produce Nic311 generally produce serum antibodies with $K_a$ values of 30 to 50 nM, and vaccination has been shown to attenuate nicotine self-administration in rats (LeSage et al., 2006) and possibly promote smoking cessation in humans (Hatsukami et al., 2005; Maurer et al., 2005). The ability to administer high doses of a monoclonal antibody and to produce serum antibody levels greatly exceeding those reported after vaccination could allow passive immunization to be even more effective than vaccination. A recent report found that a monoclonal nicotine-specific
antibody administered at a dose of 50 mg/kg was slightly more effective than vaccination with the same immunogen for reducing locomotor activity in rats (Carrera et al., 2004). Although serum antibody concentrations were not measured, these results are consistent with the potentially greater efficacy of passive immunization compared with vaccination.

Studies of monoclonal antibodies to phencyclidine have shown that doses as low as 15 mg/kg (equivalent to just 1% of the phencyclidine body burden) reduced chronic phencyclidine accumulation in brain and phencyclidine-induced locomotor activity (Laurenzana et al., 2003b). This pharmacokinetic efficacy seems to be greater than that of Nic311 in the current study. The low $K_0$ of 1.3 nM of the phencyclidine antibody may have contributed to its efficacy, but Nic-IgG, with a $K_0$ of 1.6 nM, was not appreciably more effective in the current study than Nic311, with a $K_0$ of 60 nM. These data suggest that factors beyond antibody affinity and dose, such as the pharmacokinetics of the target drugs themselves, influence the efficacy of immunization.

Serum levels of the nicotine metabolite cotinine are commonly used as a measure of nicotine intake in smokers. Although the binding of nicotine by Nic311 reduces nicotine clearance 10-fold and more than doubles its elimination half-life (Keyler et al., 2005b), serum levels of cotinine were not altered by antibody treatment in this study. Possibly, a small amount of binding of cotinine to Nic311 increased cotinine retention in serum. If this also occurs in humans, then cotinine might be useful even in immunized individuals for estimating nicotine intake.

A limitation of this study is that higher doses of Nic-IgG (80 and 240 mg/kg) could not be used because of antibody availability. However, the effects of the 10- and 30-mg/kg doses were readily measurable and allowed comparison with the equivalent doses of Nic311. The duration of nicotine dosing of 40 h was also somewhat short as a model of chronic dosing. A nicotine elimination half-life of 2.5 h has been reported in rats treated with 10 mg/kg Nic311 (Keyler et al., 2005b), so that 16 h would be sufficient to approximate steady-state nicotine levels in the presence of antibody. This half-life is probably longer in rats receiving higher Nic311 doses so that true steady-state conditions might not have been achieved, and studies of longer duration might be of interest.

These data support a potential role for passive immunization with monoclonal antibodies in the treatment of tobacco dependence. The $K_0$ of Nic311 is similar to that elicited by vaccination of rats against nicotine with the same immunogen, and higher serum antibody concentrations can be reduced by passive immunization than by vaccination. A therapeutically monoclonal antibody would need to be humanized to provide advantages such as greater efficacy and immediacy of onset of action.

Acknowledgments

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