Cecal Ligation and Puncture Peritonitis Model Shows Decreased Nicotinic Acetylcholine Receptor Numbers in Rat Muscle

Immunopathologic Mechanisms

Hiroshi Tsukagoshi, M.D.,* Toshihiro Morita, M.D.,† Kenichiro Takahashi, M.D., PhD,‡ Fumio Kunimoto, M.D.,§ Fumio Goto, M.D.¶

Background: Although systemic inflammation is believed to cause upregulation of nicotinic acetylcholine receptors (nAchRs) in muscle, chronic infections such as Chagas' disease occasionally are complicate by myasthenia gravis. The authors investigated how a nonlethal cecal ligation and puncture (CLP) peritonitis model in rats could affect muscle nAchR.

Methods: On day 1, 4, 7, 14, or 21 after CLP or sham operation, nAchR binding was assayed in the anterior tibial muscle and diaphragm using [125I]-bungarotoxin. The presence or absence of weakness, in vivo dose-response relationships for d-tubocurarine, and serum anti-nAchR antibody titers were assayed in separate experiments.

Results: Systemic inflammation was most severe during the first 4 to 5 days. Numbers of nAchRs were decreased in anterior tibial muscle on days 7, 14, and 21 after CLP, and in the diaphragm on days 7 and 14 (P < 0.01). Both 50% and 90% blocking doses of d-tubocurarine were lower in CLP rats than in sham-operated rats on days 7, 14, and 21 (P < .05). Weakness was overt in approximately half of CLP rats at these times. Serum anti-nAchR antibody (0.7–1.4 ms) was detectable beginning on day 4 and continuing throughout the 21-day observation period in 58–67% of CLP rats.

Conclusions: During the recovery phase of injury, nonlethal CLP peritonitis resulted in downregulation of nAchR. However, further study is needed to determine the role of anti-nAchR antibodies in the development of decreased receptor numbers and impaired neuromuscular function. (Key words: Acetylcholine receptor; antinicotinic acetylcholine receptor; autoantibody; β-glucuronidase; neuromuscular relaxant; nicotinic; systemic inflammation; d-tubocurarine.)

UPREGULATION (increases in number) of muscle nicotinic acetylcholine receptors (nAchRs) is found in a variety of conditions, including denervation, disuse atrophy, burns, long-term anti-convulsant treatment, and pharmacologic pre-or postsynaptic blockade. Uprgulation usually is associated with hyposensitivity to nondepolarizing neuromuscular drugs such as d-tubocurarine (dTc) and hypersensitivity to the depolarizing drug succinylcholine associated with development of hyperkalemia. Similar abnormal responses to both types of neuromuscular blocking drugs have been reported in patients with critical illness, intraabdominal infections, massive muscle trauma, or tetanus. Dodson et al.

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drugs were studied, numbers of nAchRs in muscle were within the normal range but decreased by 37%. Possible explanations for this decrease included increased breakdown of nAchRs after initial upregulation. Systemic inflammation is believed to cause upregulation of muscle nAchRs.

Tomera and Martyn have reinforced the impression of upregulation, demonstrating that chronic intraperitoneal treatment of mice with a sublethal dose of endotoxin produces resistance (hyposensitivity) to dTc. However, numbers of nAchRs were not determined. In addition, although endotoxin is widely used as an experimental trigger of the septic response, the applicability to human disease has been questioned. Multiple exposures to sublethal doses of endotoxin are known to induce tolerance to this toxin. Clinical systemic inflammation usually is initiated or amplified by infection and evolves over many days, a time frame that is much longer than studies of the effects of endotoxin administration (1-6 h). Thus, typical endotoxin models may not reflect most clinical situations.

If infection and subsequent systemic inflammation are subacute or prolonged, autoantibody production directed to several molecular constituents frequently is seen. In some circumstances, organ-specific autoantibodies such as anti-nAchRs antibody are found. Chagas' disease (American trypanosomiasis) is the best example of how a chronic infection produces anti-nAchR antibody in association with neurotransmission failure. Patients with acquired immunodeficiency syndrome sometimes harbor anti-nAchR antibody and manifest clinical features of myasthenia gravis (MG). These observations suggest that particular infectious diseases and subsequent systemic inflammation may produce downregulation of muscle nAchR, and the possibility of infections as causative agents in MG has been raised.

The present study in rats tested the hypothesis that systemic inflammation induced by a very mild degree of cecal ligation and puncture (CLP) peritonitis could induce downregulation of muscle nAchR through immunopathologic mechanisms. The CLP peritonitis model causes prolonged bacterial infections with mixed intestinal flora resembling clinical peritonitis. Three series of experiments were performed. In initial studies, nAchR binding was assayed in anterior tibial muscle and diaphragm using [125I]-α-bungarotoxin after CLP. In the second series of experiments, presence or absence of weakness and in vivo dose-response relationships for dTc in anterior tibial muscle were evaluated. Finally, the immunopathologic hypothesis was tested by measuring serum anti-nAchR antibody titers.

Materials and Methods

This experimental protocol was approved by the Animal Care and Use Committee of Gunma University School of Medicine. Male Wistar rats (Japan Laboratory Animals, Tokyo, Japan) weighing 290-340 g were used. The animals were housed in groups of 2 or 3 in aluminum cages and were freely given a solid diet (MF; Oriental Yeast, Tokyo, Japan) and tap water. The breeding room was controlled to maintain a light-dark cycle with a light period between 6 AM and 6 PM. The temperature was kept constant at 23 ± 2°C.

The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) followed by incremental doses as necessary. Under sterile conditions, the abdomen of the rat was shaved and a 2.0- to 2.5-cm midline incision was made. The cecum was exteriorized and filled with feces by milking stool back from the ascending colon. The cecum was ligated with a 3-0 silk ligature just below the ileocecal valve without causing bowel obstruction and punctured once with a 24-gauge needle. Gentle pressure was applied on the ligated cecum to exteriorize a small amount of feces. The cecum was returned to the peritoneal cavity, and the abdomen was closed in two layers with a 3-0 silk suture. Sham-operated rats received the same anesthesia and surgical manipulation without cecal ligation and puncture. After surgery, both groups of rats received a single dose of saline subcutaneously (3 ml/100 g body weight) for fluid resuscitation and were heated by a warming lamp for approximately 3 h. Rats were weighed daily.

Part 1: Numbers of nAchRs after CLP

Tissue and Blood Samples. On days 1, 4, 7, 14, or 21 after CLP or sham operation, rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). The left anterior tibial muscle was removed surgically and washed in 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 2 mM benzamidine hydrochloride, 0.1 mM phenylmethylsulphonyl fluoride, 0.5 mg/ml bacitracin, and 0.02% sodium azide. After weighing, it was stored at −80°C until nAchR assay. Three to four milliliters of blood were withdrawn from the abdominal aorta into heparinized syringes and were transferred to sterile glass tubes for β-glucuronidase assays. Blood samples were immediately centrifuged at 1000 g for 10 min at 4°C, and the plasma was stored at −80°C. The diaphragm was excised and prepared in a manner similar to the left anterior tibial muscle. In some rats, on days 4, 7,
and 14 (n = 5–7 for each group on each day), the right anterior tibial muscle was excised for cyclic adenosine monophosphate (cAMP) assay before blood sampling and excision of the left anterior tibial muscle. Muscle for the cAMP assay was immediately frozen in liquid nitrogen and stored at -80°C.

**Acetylcholine Receptor Assay.** Muscle-membrane preparation and determination of nAchR numbers were performed using a modification of the methods proposed by Kim et al.8 Frozen muscle samples were thawed, finely chopped, and homogenized in 4 volumes of 10 mM potassium phosphate buffer. After the homogenate was centrifuged (20,000g for 30 min), the resulting supernatant was decanted and discarded. The pellet was resuspended in the same buffer (1 vol) additionally containing 2% (v/v) Triton X-100 (Sigma Chemical Company, St. Louis, MO), a detergent that extracts nAchR. This extract was shaken overnight (approximately 14 h) in a cold room (4°C) and then centrifuged at 20,000g for 45 min at 4°C. The supernatant was used for the nAchR assay.

To determine nAchR number, [125I]α-bungarotoxin (specific activity, 16.2 600 kBq/μg; NEN, Boston, MA) was used. This ligand binds specifically and irreversibly to the α-subunit of nAchR. Triplicate or duplicate samples of crude muscle extract in Triton buffer (approximately 0.5 mg protein per tube, final volume 1 ml) were mixed with 2.5 nm [125I]α-bungarotoxin for 90 min at room temperature (23°C). Reactions were terminated by addition of 3 ml of ice-cold Triton buffer, after which bound radioligand was separated by filtration using Whatman DE-81 cellulose filters (Whatman International Ltd., Maidstone, United Kingdom). The filters were washed three times with ice-cold Triton buffer, and air-dried before counting in a γ counter (ARC-300, Aloka, Tokyo, Japan). Specific binding of [125I]α-bungarotoxin was defined as the difference between the binding in the absence and in the presence of 1 μM unlabeled α-bungarotoxin (Sigma Chemical Co., St Louis, MO). Numbers of nAchRs were expressed as femtomoles per milligram protein.

**Determination of Protein Concentration.** Protein concentrations were determined using protein assay kits (Pierce Laboratories, Rockford, IL), according to the bicinchoninic acid method,52 using bovine plasma albumin as a standard. Diluted samples (0.1 ml; 1:1 and 1:3) were incubated with 2 ml of the protein reagent for 30 min at 37°C. Absorbance was measured at 562 nm. Intraassay variation was 2.5%. The lower limit of sensitivity was 0.02 mg/ml.

**Determination of cAMP in the Anterior Tibial Muscle.** Cyclic adenosine monophosphate in the anterior tibial muscle was measured to elucidate the effects of CLP injury on skeletal muscle. Frozen samples were powdered in liquid nitrogen using a porcelain mortar. The powder was extracted with 10 volumes of 2 mM EDTA acidified with 0.1-N HCl, and the extract was boiled for 10 min in a water bath. After centrifugation (3000 rpm, 10 min), the supernatant was assayed for cAMP using a cAMP radioimmunoassay kit (Yamasa Shoyu, Choshi, Japan).53 Intra- and interassay variation were 4% and 6.5%, respectively. The lower limit of sensitivity was 0.1 pm. Data were expressed as picomoles per milligram protein.

**Plasma β-Glucuronidase Concentrations.** As a marker of cell-membrane damage,54,55 level of plasma β-glucuronidase, a lysosomal enzyme, was determined with phenolphthalein glucuronic acid as substrate according to Fishman et al.56 Plasma (0.1 ml) was incubated with 0.1 ml substrate solution for 20 h at 37°C. The reaction was terminated by adding 2 ml ice-cold glycine-NaOH buffer (0.1 μ, pH 10.5), containing 5 mM EDTA disodium. The fluorescence of the liberated substrate was measured at 450 nm. Activity was expressed as units per 100 milliliters of plasma (Fishman units), where 1 unit hydrolyzes 1 μmol of substrate per hour. Intraassay variation was 6%. The lower limit of sensitivity was 20 U/100 ml plasma.

**Part 2: Forced Exercise Test and dTc Dose-Response Study**

Additional groups of rats (n = 5–12 for each) were used. On days 1, 4, 7, 14, and 21 after CLP or sham operation, rats were observed behaviorally. They were subjected to forced exercise in which they were allowed to climb up a screen and then stand on its top edge, or if the rat could maintain the vertical position before that time. The procedure was repeated four times in rapid succession. If a rat developed an abnormal behavioral response during this procedure or upon its completion, the animal was regarded as showing weakness.
dTc Dose–Response Study. The same rats were reanesthetized with pentobarbital intraperitoneally. After tracheostomy, the animals were ventilated with 50% O₂ using a Harvard respirator to produce normal arterial CO₂ values. Polyethylene catheters were inserted in the right carotid artery for continuous measurement of blood pressure and for blood-gas analysis, and in the jugular vein for dTc and fluid administration. Blood (0.3 ml) was withdrawn from the carotid artery for blood-gas and hemoglobin determinations (ABL 300, Radiometer, Copenhagen, Denmark). Blood withdrawn was replaced with normal saline (0.6 ml). Arterial electrolyte (Na⁺, K⁺, Cl⁻, and Ca²⁺) and glucose levels were determined (Ciba-Corning 860, Chiron, MA) in most rats. Rectal temperature was maintained between 36 and 37°C with a warming pad and recorded using a rectal thermometer.

The left sciatic nerve was isolated in the gluteal region, placed on bipolar stainless electrodes, and crushed with a heavy ligature proximal to the electrodes. The knee was stabilized rigidly in a clamp. The distal tendon of the left tibialis anterior muscle was dissected, separated from its insertion, and attached to a TB-611T force transducer (Nihon Koden, Tokyo, Japan). The preload was adjusted to 50 g in the sham-operated group, but only to 20 g in the CLP group, to minimize effects of CLP injury on the twitch response. Supramaximal stimuli of 0.15 Hz in frequency and 0.2 ms in duration (Electronic Stimulator SEN-3301, Nihon Koden) were delivered on the sciatic nerve, and twitch response and blood-pressure data were recorded on a continuously running recorder (Nihon Koden). If the twitch response was stable for at least 10 min, incremental doses of dTc (first two doses, 20 μg/kg; subsequent doses, 10 μg/kg) were administered via the jugular vein to achieve 95 to 100% twitch depression. Cumulative dose–response curves were constructed by linear regression of the logarithm of the dose against a logit transformation of the percentage suppression of twitch responses, from which the effective doses of dTc for suppression of twitch response by 50% and 90% of control (ED₅₀ and ED₉₀) were derived. If maximal recovery of twitch tension was observed, rats were killed by intravenous overdoses of pentobarbital.

Part 3: Anti-nAChR Antibody Assay

After pentobarbital anesthesia (50 mg/kg intraperitoneally), 3–4 ml of blood was collected from the abdominal aorta for anti-nAChR antibody assay. The sample was centrifuged at 4000g for 20 min at 4°C and was stored at −80°C. Sera from CLP rats (n = 6–12 on each day) and sham-operated rats (n = 5 on each day) were tested for anti-nAChR antibodies by a radioimmunoassay using denervated rat-leg nAChR as an antigen according to the method of Patrick et al. This assay measures IgG antibody directed against sites on the nAChR molecule other than the acetylcholine binding site. Denervation was performed at the thigh by removal of a 1-cm segment of the sciatic nerve 14 days previously (n = 16). The methods used for denervated rat-leg nAChR solubilization and analysis were the same as in part 1 of the study. Numbers of nAChR in the denervated muscle were 112 ± 14 fmol/mg protein (0.55–0.75 pmol/ml). Three sera with known antibody concentrations (3–4 nm; RSR, Cardiff, United Kingdom) from human myasthenia patients were used as a positive control. Denervated rat nAChR have been demonstrated to react with human anti-nAChR antibodies, although the degree of reactivity varies among MG patients. Each tube (in duplicate) was prepared containing 80 fmol of the receptor, fivefold molar excess of [¹²⁵I]α-bungarotoxin over receptor, 10 μl of normal rat serum, and 1 μl of serum from CLP rats, sham-operated rats, or human myasthenic patients. Each tube was then made to contain 175 μl of reaction mixture by addition of Triton buffer. After overnight incubation at 4°C, 25 μl of goat anti-rat immunoglobulin G antiserum (ICP Pharmaceuticals, Aurora, OH) was added; for human MG sera, goat anti-human immunoglobulin G antiserum (ICP Pharmaceuticals) was used. After incubation for 7 h at 4°C, the immune precipitate was sedimented by centrifugation and the resulting pellet was washed twice with 1 ml Triton buffer. Radioactivity trapped in the pellet was measured using a γ counter. Nonspecific binding was defined as radioactivity trapped in the pellet in the presence of dTc (1 nm). Control assays also used [¹²⁵I]α-bungarotoxin without receptor in the presence of serum, or without any serum in the presence of receptor. Titer of anti-nAChR antibody were expressed as nanomoles per liter.

Statistical Analysis. Results are expressed as mean ± SD. One-way analysis of variance was used to test differences within groups. The unpaired t test was used to compare differences between groups. A chi-square test was used for the analysis of the presence or absence of anti-nAChR antibodies. A P value less than 0.05 was considered statistically significant.

Results

Part 1

Mortality, Behavior, and Weight Gain. No deaths occurred in sham-operated rats (n = 31). Because of
greater risk of death in CLP rats, more rats were used (n = 51). All deaths associated with CLP (5 of 51 rats; 9.8%) occurred 2-5 days after CLP. At necropsy of these animals, the peritoneal cavity contained a large amount of bloody, malodorous fluid, and the cecum was distended and gangrenous. The remaining 46 rats were studied as the CLP group. Surviving CLP rats developed diarrhea and failed to gain weight (4.5% of weight loss on day 4). They also showed crouching, piloerection, and decreased spontaneous locomotor activity. From days 5-7, these manifestations of illness began to disappear, and the rats gained weight, paralleling the weight gain in sham-operated rats. *P < 0.05, **P < 0.01 compared with sham-operated rats.

**Number of nAchRs.** Protein concentrations of muscle membrane used for nAchR assay did not differ between groups in either anterior tibial muscle (3.71 ± 0.3 mg/ml) or diaphragm (3.20 ± 0.4 mg/ml) throughout the observation period (P > 0.05). Nonspecific binding of [125I]α-bungarotoxin also was similar between groups at each time point. Nonspecific binding of total binding was relatively high (65 ± 2.3% in the anterior tibial muscle and 63 ± 3.3% in the diaphragm). Changes in nAchR number are summarized in table 1. In the sham-operated rats, no remarkable changes in number of nAchRs occurred in either muscle tested throughout the 21-day observation period (P > 0.05). Although on days 1 and 4 the numbers of nAchR in both anterior tibial muscle and diaphragm showed no difference between groups, receptor numbers were lower in CLP rats than in sham-operated rats (P < 0.01) on days 7 and 14. At 21 days, a significant difference persisted in number of nAchRs in the anterior tibial muscle between CLP and sham-operated rats (P < 0.01), but the difference in the diaphragm no longer was significant (table 1).

To assess lower limit of detection sensitivity, pooled samples from anterior tibial muscles of four sham-operated rats were serially diluted from 1 mg protein to 0.125 mg protein with Triton buffer. Each dilution was assayed for nAchR number in duplicate six to eight times. The nAchR numbers per tube and mean coefficients of variation in parentheses were 7.02 ± 1.2 fmol (16.9%) at 1.0 mg protein, 3.59 ± 0.6 fmol (16.8%) at 0.5 mg protein, 2.48 ± 1.2 fmol (48.3%) at 0.33 mg protein, 2.41 ± 1.4 fmol (57%) at 0.25 mg protein, and 2.22 ± 1.1 fmol (51%) at 0.125 mg protein. The measured nAchR numbers were approximately proportional to the dilution at the protein concentrations of 1.0, 0.5, and 0.33 mg, but not at protein concentrations less than 0.25 mg. The lower limit of detection was 2.3 fmol/mg protein. The method used in the present study was unreliable in assaying very low levels of nAchR.

**Anterior Tibial Muscle cAMP.** As shown in table 2, higher cAMP levels were found in CLP rats than in sham-operated rats on days 4, 7, and 14 (P < 0.01 on days 4 and 7; P < 0.05 on day 14).

**Plasma β-Glucuronidase Concentrations.** As described in table 3, higher plasma β-glucuronidase concentrations were observed in CLP rats than in sham-operated rats on days 4 and 7 (P < 0.01 on day 4; P < 0.05 on day 7). On day 21 after CLP, plasma β-glucuronidase concentrations were as low as in sham-operated rats (table 3).

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PERITONITIS DECREASES MUSCLE NICOTINIC RECEPTORS

Table 1. Numbers of nAchR (fmol/mg Protein) in Anterior Tibial Muscle and Diaphragm after CLP

<table>
<thead>
<tr>
<th>Group</th>
<th>A. Anterior tibial muscle</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td>6.13 ± 1.4 (n = 5)</td>
<td>5.62 ± 1.3 (n = 7)</td>
<td>6.23 ± 1.9 (n = 6)</td>
<td>6.45 ± 2.2 (n = 7)</td>
<td>6.64 ± 1.3 (n = 6)</td>
</tr>
<tr>
<td>CLP</td>
<td></td>
<td>6.65 ± 1.6 (n = 7)</td>
<td>5.17 ± 1.9 (n = 6)</td>
<td>2.60 ± 1.0 (n = 12)</td>
<td>2.61 ± 1.0 (n = 14)</td>
<td>3.89 ± 1.7* (n = 7)</td>
</tr>
<tr>
<td>B. Diaphragm</td>
<td></td>
<td>8.83 ± 3.0 (n = 5)</td>
<td>7.30 ± 2.0 (n = 7)</td>
<td>7.50 ± 1.8 (n = 6)</td>
<td>8.61 ± 3.9 (n = 7)</td>
<td>8.53 ± 1.5 (n = 6)</td>
</tr>
<tr>
<td>Sham</td>
<td></td>
<td>7.52 ± 1.1 (n = 7)</td>
<td>7.56 ± 3.5 (n = 6)</td>
<td>2.34 ± 1.3* (n = 12)</td>
<td>2.12 ± 1.1* (n = 14)</td>
<td>5.87 ± 3.5 (n = 7)</td>
</tr>
<tr>
<td>CLP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD.
CLP = cecal ligation and puncture; Sham = sham operation.
*P < 0.01 versus sham-operated rats at corresponding days.

Part 2

Forced Exercise Test. Body-weight changes, mortality rate (12%), and behavior were essentially comparable with observations in part 1. Numbers of rats showing weakness and abnormal postures are presented in Table 4. Sham-operated rats showed no weakness throughout the observation period. On days 1 and 4 after CLP, only two to three rats exhibited weakness. However, on days 7, 14, and 21, weakness was overt in approximately half of CLP rats. CLP rats showing abnormal postures always had weakness (Table 4).

Alterations in brain function sometimes are observed during severe systemic inflammation.59 However, none of the CLP rats showed characteristic signs of septic encephalopathy such as loss of interest in surroundings or delay in the foot withdrawal reaction in response to toe pinching.59 In addition, the CLP rats showing an abnormal response to forced exercise nonetheless attempted to maintain the vertical position. These observations suggest that abnormal responses in this test were not caused by brain dysfunction.

Dose–Response Study. In most CLP rats, twitch tension stabilized within 10 min throughout the 21-day observation period, although slight decreases in twitch response were observed in some instances. However, in two to three CLP rats on days 7, 14, and 21, despite use of only 20 g of tension, the twitch responses exhibited a progressive decline to near-baseline levels. This abnormal response was not observed in sham-operated rats, or even in CLP rats on days 1 and 4. Interruption of sciatic nerve stimulation for 15 to 20 min, repositioning the electrodes, or increasing the output of stimulating current could not produce normalization of this response. Because of concern that this progressive decline could be related to technique, twitch tracings were obtained in the contralateral leg, but these were similar to those in the first leg tested. In these animals, the dose–response study was aborted.

During the stabilization period for twitch tension, cardiovascular variables, arterial blood-gas measurements, electrolyte concentrations (Na⁺, K⁺, Cl⁻, and Ca²⁺), and glucose levels did not differ between groups throughout the 21-day observation period. However, relatively high hemoglobin concentrations were observed on days 1 and 4, and in some instances through day 7. Administration of dTc frequently produced more severe hypotension and bradycardia in CLP rats at these time points, and occasionally also on days 14 and 21. Accordingly, intervals between doses were lengthened in these rats. However, more deaths occurred during the dose–response study in CLP rats than in sham-operated rats. Because of death from cardiovascular collapse induced by dTc and observation of abnormal tracings, only six or seven rats completed the dose–response study in the CLP group for each time point.

Representative twitch and blood-pressure tracings

Table 2. Concentrations of cAMP in the Anterior Tibial Muscle (pmol/mg Protein)

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.39 ± 0.38 (n=5)</td>
<td>1.45 ± 0.33 (n=5)</td>
<td>1.48 ± 0.31 (n=5)</td>
</tr>
<tr>
<td>CLP</td>
<td>2.19 ± 0.41* (n=6)</td>
<td>2.06 ± 0.23* (n=7)</td>
<td>1.79 ± 0.16* (n=7)</td>
</tr>
</tbody>
</table>

Values are mean ± SD.
CLP = cecal ligation and puncture; Sham = sham operation.
*P < 0.01 versus sham at corresponding days.
†P < 0.05 versus sham.
from sham-operated and CLP rats on day 14 are shown in figure 2. Estimated ED$_{50}$ and ED$_{90}$ values are presented in figure 3. On days 1 and 4, neither ED$_{50}$ nor ED$_{90}$ values differed between groups. On days 7, 14, and 21, both ED$_{50}$ and ED$_{90}$ values were significantly lower in CLP rats than in sham-operated rats ($P < 0.05$).

### Table 3. Changes in Plasma β-Glucuronidase Concentrations (Fishman units/100 ml)

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>328.9 ± 72.0</td>
<td>280.7 ± 116.9</td>
<td>358.6 ± 134.2</td>
<td>312.9 ± 37.0</td>
<td>289.0 ± 69.7</td>
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<tr>
<td>CLP</td>
<td>499.4 ± 163.8</td>
<td>623.9 ± 95.7</td>
<td>580.2 ± 163.9</td>
<td>475.0 ± 139.3</td>
<td>247.3 ± 80.1</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

CLP = cecal ligation and puncture; Sham = sham operation.

* $P < 0.01$, †$P < 0.05$ versus sham-operated rats.

### Part 3

**Anti-acetylcholine Receptor Antibody.** To determine the optimal nAchR concentration for use as antigen for the anti-nAchR antibody assay, three sera from CLP rats were tested in duplicate with different concentrations (40, 80, 120, and 150 fmol) of receptors. We found that at any nAchR concentration greater than 80 fmol, no differences in the amount of precipitated $[^{125}]$α-bungarotoxin-nAchR complex were apparent. Thus, we always used the nAchR concentration of 80 fmol.

In the absence of receptor, or in the absence of any serum, the amount of precipitated $[^{125}]$α-bungarotoxin was very small (150–210 cpm). Sera from sham-operated rats caused precipitation that differed only slightly from the amount of $[^{125}]$α-bungarotoxin-nAchR complex nonspecifically trapped in the dTc-treated pellet. This value did not differ between the five time points for sham-operated rats ($n = 5$ per time point). The five time points for sham-operated rats were combined for comparison with CLP rats and are represented in table 5 (0.24 ± 0.11 nm). Significantly higher titers of anti-nAchR antibody first were detected in sera from CLP rats on day 4 (0.80 ± 0.28 nm). They were present throughout the 21-day observation period (table 5). Values above the mean plus three SDs for the sham-operated group (0.7 nm) were considered positive. Serum anti-nAchR antibodies were present in 58 to 67% of CLP rats between days 4 and 21. However, no differences in either antibody titers or positive frequency were found between the CLP groups at these time points. Anti-nAchR antibody was detectable in human positive control serum at appropriate levels in three independent analyses (range, 2.1–5.4 nm).

Three positive sera (1.4, 1.2, and 1.0 nm) from CLP rats were assayed in duplicate for six times to assess within-assay precision. The mean coefficient variations ranged from 9.6 to 14.6% (mean, 11.9%). These sera were serially diluted in normal rat sera (1:1, 1:2, and 1:3) and were assayed in duplicate three times to assess the lower limit of detection. The recoveries of serum dilutions (i.e., anti-nAchR antibody measured/expected) ranged between 88% and 136%. The lower limit of sensitivity for anti-nAchR antibody was 0.25 nm.

### Table 4. Weakness and Abnormal Postures

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Weakness*</th>
<th>Abnormal Postures†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>26t</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 day post-CLP</td>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>4 days post-CLP</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>7 days post-CLP</td>
<td>11</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>14 days post-CLP</td>
<td>11</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>21 days post-CLP</td>
<td>12</td>
<td>6</td>
<td>6</td>
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</tbody>
</table>

CLP = cecal ligation and puncture; Sham = sham operation.

* Numbers of rats showing weakness as evaluated by the forced exercise test.

† Numbers of rats showing abnormal postures such as a lowered head, a hunched back, and partially abducted thighs.

‡ Three sham-operated rats died during dose response study.

### Discussion

The most important findings of this study were obtained during the recovery phase of CLP-induced systemic inflammation: These were that (1) numbers of nAchRs in both anterior tibial muscle and diaphragm were reduced in CLP rats but not in sham-operated rats; (2) the ED$_{50}$ and ED$_{90}$ values of dTc were decreased in CLP rats but not in sham-operated rats; (3) approximately half of CLP rats developed weakness; and (4) low titers of anti-nAchR antibody arose before these findings were obtained and persisted.
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Fig. 2. Representative twitch and blood pressure tracings from a sham-operated (upper panel) and a cecal ligation and puncture (CLP) rat (lower panel) on day 14. Effective doses of d-tubocurarine for twitch inhibition in the CLP rat are smaller than in the sham-operated rat. More profound hypotension after d-tubocurarine is seen in the CLP rat than in the sham-operated rat.

throughout the 21-observation period in 58–67% of CLP rats.

Relationships between Systemic Effects of CLP Injury and Downregulation of nAchR

The CLP model can reproducibly induce varying degrees of injury by varying the number and size of cecal punctures in rats.29 The present study used a very mild form of CLP with a survival rate of 90% at 21 days after the insult. Weight loss (4.5%) occurred for the first 4 to 5 days. The lysosomal enzyme β-glucuronidase was increased in plasma, peaking between days 4 and 7 but representing a smaller peak than that seen in endotoxin shock. In addition, enzyme levels returned to normal between days 14 and 21 (table 2). These results indicate that although systemic inflammation was most severe for the first 4 to 5 days after CLP, it was not profound or prolonged. However, based on necropsy findings (small amounts of cloudy fluid in the peritoneal cavity, small abscesses at the puncture site, and occasional microabscesses), chronic infections were present in the peritoneal cavity throughout the 21-day observation period. The CLP protocol used in the present study produced particularly persistent inflammation in the peritoneal cavity. Most likely as a result of the net effect of many endogenous agents released during CLP injury, cAMP concentrations in the anterior tibial muscle were elevated in CLP rats on days 4, 7, and 14 (table 3).

The numbers of nAchR in both anterior tibial and diaphragm muscles first showed reduction on day 7. The downregulation persisted at day 21 in the anterior tibial muscle, but in the diaphragm the number of nAchR returned to near-baseline levels (table 1). However, these findings should be viewed in the context of the problems involved in our nAchR binding assay (a high degree of nonspecific binding and unreliability in detecting low numbers of nAchR). In addition, to our knowledge, no other reports have shown downregulation of muscle nAchR during systemic inflammation. Rather, previous studies have reported that other forms of systemic inflammation (burn injury6–8 and critical illness21) increase nAchR numbers in muscle, although upregulation seen in critical illness may mainly reflect effects of...
Fig. 3. Estimated effective doses (in micrograms per kilogram; mean ± SD) of d-tubocurarine for suppression of twitch response by 50% and 90% of control (ED50 and ED90; in micrograms per kilogram; mean ± SD) in rats after cecal ligation and puncture (filled column) and sham operation (open column). *P < 0.05, compared with sham-operated rats.

Behavior, Decreased Locomotor Activity, and Dose Requirement of dTc

Loss of functional nAchR impairs neuromuscular transmission to result in weakness, hypersensitivity to nondepolarizing neuromuscular blocking drugs, and a decremental electromyographic response after repeated nerve stimulation. In the present study, dTc was used as a test drug because it is the best understood pharmacologic probe of the neuromuscular junction. After burn injury or partial denervation, an increase in numbers of nAchR shows a positive correlation with an increase in the effective dose of dTc for twitch inhibition. Conversely, decreased nAchR in MG results in increased sensitivity to dTc.

The decreased ED50 and ED90 values observed on days 7, 14, and 21 after CLP are compatible with the finding that numbers of nAchR are significantly reduced in the anterior tibial muscle at these times. In fact, the numbers of nAchR in CLP rats sometimes were below the lower limit of detection by the method used in the present study. Although prolonged effects of vecuronium have been described in patients with acquired immunodeficiency syndrome, our results contradict numerous previous studies that state that systemic inflammation is associated with decreased sensitivity to nondepolarizing neuromuscular drugs. These include burn injury, critical illness, and chronic treatment with endotoxin in mice. In the present study, arterial concentrations of electrolytes (Na+, K+, Cl−, and Ca2+) and arterial blood-gas levels did not differ between groups throughout the 21-day observation period. On days 1 and 4, at the acute phase of CLP injury, the ED50 and ED90 values were normal but somewhat high. These results suggest that effects of systemic inflammation were not pronounced enough to significantly affect sensitivity to dTc. However, arterial electrolyte levels were not measured in all animals, and no attempt was made to determine the levels of ionized magnesium, which is of interest because hypermagnesemia causes neurosuppression. Firm conclusions cannot be drawn regarding

Table 5. Serum Anti-nAchR Antibody Titers and Its Positive Frequency

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Anti-nAchR Antibody [nM]</th>
<th>Positive Frequency [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>25</td>
<td>0.24 ± 0.11</td>
<td>0</td>
</tr>
<tr>
<td>1 day post-CLP</td>
<td>6</td>
<td>0.22 ± 0.09</td>
<td>0</td>
</tr>
<tr>
<td>4 days post-CLP</td>
<td>11</td>
<td>0.80 ± 0.29$\uparrow$</td>
<td>64</td>
</tr>
<tr>
<td>7 days post-CLP</td>
<td>12</td>
<td>0.70 ± 0.30$\uparrow$</td>
<td>67</td>
</tr>
<tr>
<td>14 days post-CLP</td>
<td>12</td>
<td>0.77 ± 0.29$\uparrow$</td>
<td>58</td>
</tr>
<tr>
<td>21 days post-CLP</td>
<td>11</td>
<td>0.71 ± 0.26$\uparrow$</td>
<td>64</td>
</tr>
</tbody>
</table>

Values are mean ± SD.
CLP = cecal ligation and puncture; Sham = sham operation.
* Serum anti-nAchR (nicotinic acetylcholine receptor) antibody titers were measured by a radioimmunoassay using denervated rat leg muscle nAchR as an antigen.
† Values above 0.7 nM are considered positive (> mean ± 3SD for the sham-operated rats).
‡ The five groups of sham-operated rats (n = 5 on each day) are combined for comparison with CLP rats.
§ P < 0.01 versus the values in sera from sham rats.
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complete normality of electrolyte concentrations during the course of CLP injury. Furthermore, systemic inflammation frequently is associated with dehydration, liver and renal dysfunction, and hypoproteinemia. All of these factors decrease the amount of dTc required to produce an effect, both by decreasing the elimination rate of dTc and by increasing the free fraction of plasma dTc. Thus, the present study did not rule out the possibility of alterations in pharmacokinetic or pharmacodynamic mechanisms as contributing to the decreased ED₅₀ and ED₉₀ values. A pharmacodynamic investigation of the relationship between plasma dTc concentrations and a given level of twitch suppression is required to rule out alterations in pharmacokinetic mechanisms.

The weakness and decreased locomotor activity observed in CLP rats on days 1 and 4 were largely caused by effects of systemic inflammation induced by CLP injury. However, CLP rats showing weakness between days 7 and 21 frequently also showed abnormal postures such as a lowered head, a hunched back, and partially abducted thighs (table 4). These abnormal postures were not observed on days 1 and 4. In an animal model of MG produced by immunization with purified nAChR, experimental autoimmune MG (EAMG), similar abnormal postures are seen. In EAMG, these signs are characteristic of weakness secondary to downregulation of muscle nAChR. Disuse atrophy secondary to long-term immobilization also results in weakness but is unlikely to have contributed to observations between days 7 and 21, because disuse is known to cause upregulation of nAChR associated with decreased sensitivity to nondepolarizing neuromuscular drugs. Based on the decreased number of nAChRs, the presence of antibodies, and the increased sensitivity to dTc, we considered nAChR loss to be at least partly responsible for the weakness, abnormal postures, and decreased spontaneous locomotor activity observed between days 7 and 21.

However, nAChR loss can not in itself account for the spontaneous decline in the single twitch response observed on days 7, 14, and 21 after CLP, although the forced exercise test prior to the dose-response study might have augmented fatigue. Even in severely affected EAMG rats, a decremental electromyographic response can be seen only after repeated nerve stimulation. Tomera and Martyn reported that malnutrition (a 5% protein diet for 3 weeks) produces a spontaneous decline in the single twitch response with a concomitant increase in cAMP levels in mouse muscle. They have proposed that these changes are caused by metabolic changes in muscle. Some degree of malnutrition may have occurred in our rats, because even at 21 days after CLP local inflammation persisted in the peritoneal cavity, and the anatomic abnormality created in the bowel still was present. In addition, accelerated breakdown of muscle protein is a characteristic metabolic response to systemic inflammation. A component of the muscle could be involved in this abnormal response.

Possible Mechanisms of Downregulation

Initially, the possibility of a causative link was considered between the observed increase in plasma lysosomal enzyme β-glucuronidase and downregulation, because the autophagic lysosome system is considered pivotal in the degradation or turnover of muscle nAChR. Because β-glucuronidase is distributed in both lysosomes and cytosol of many tissues, however, the source of β-glucuronidase release in the present study is not known in any detail. Furthermore, the time course of the peak increased in plasma β-glucuronidase did not correlate with that of the reduction of nAChR numbers. Thus, the downregulation is unlikely to have resulted directly from activation of the muscle lysosome system.

Downregulation of nAChR is a very rare phenomenon reported only in MG and in long-term agonist stimulation. In MG, reduction of nAChR number results from a complement-mediated attack and accelerated degradation of nAChR crosslinked by antibody, nAChR reduction following agonist stimulation reflects acetylcholine-induced receptor phosphorylation. Anti-nAChR antibodies are specific for MG and its model EAMG, but not for other autoimmune diseases. Using nAChR isolated from denervated rat muscle as an antigen, significantly higher titers of IgG anti-nAChR antibody directed against the nAChR were detectable from day 4 throughout the observation period in CLP rats than in sham-operated rats (table 5). Both decreased numbers of nAChR and signs of impaired neuromuscular transmission (increased sensitivity to dTc and weakness) were first observed on day 7 (table 1). These data suggest that an anti-nAChR antibody-mediated mechanism, as in MG, might initiate or amplify the receptor changes and thereby might induce neuromuscular transmission failure. However, in EAMG, much higher titers of antibody (>10 nm) usually are found than in CLP rats (1.4 nm: maximum titer observed). In addition, serum anti-nAChR antibody was detected only in 58–67% of CLP rats between days 4 and 21. The sensitivity of the assay system used in the present study may not have been sufficient to reliably detect very low titers of antibody,
and the mean value and SD for sham-operated rats were relatively high (0.24 ± 0.11 nM). Furthermore, despite the presence of the anti-nAChR antibody in the serum, receptor numbers returned to near baseline values by day 21 in the diaphragm, but not in the anterior tibial muscle. These results indicate that the low titers of antibody observed may not be sufficient in themselves to account for development of CLP-induced neuromuscular changes, and additional factors must be at work.

Based on persistence of increased cAMP levels in anterior tibial muscle, CLP-induced downregulation is likely to be associated with increased synthesis of receptors; cAMP is known to increase expression of muscle nAChR via increased transcriptional and post-transcriptional mechanisms. This implies that an increase in receptor synthesis may be accompanied by a greater increase in breakdown to result in a net decrease in receptors. Antibody-induced modulation of nAChR is known to involve increased turnover rate of receptors. Further study is necessary to determine the relative rates of synthesis and degradation of muscle nAChR.

What initiates formation of anti-nAChR antibodies in previously normal rats that undergo a simple manipulation of the cecum? One plausible explanation is infection by the animals own intestinal flora. An essential feature underlying our model is that infections by intestinal flora are prolonged, and the animals are not able to neutralize the infectious agents. This makes persistent immune stimulation likely. Stefansson et al. have reported that intestinal bacteria (Escherichia coli, Klebsiella pneumoniae, and Proteus vulgaris) may induce autoimmune responses through shared determinants between the nAChR and bacterial proteins, by altering the host immune system, or by causing release of sequestered "self" antigens. How the CLP protocol used in the present study acts to modify the immune system is unknown and will be the subject of future studies. However, one might speculate that molecular mimicry between intestinal bacterial antigenic determinants and epitopes on the nAChR may be an initiating mechanism.

Conclusions

We showed an effect of systemic inflammation on neuromuscular function that is distinctly opposite to that found in several previous investigations: a decrease in receptor numbers in both limb and diaphragmatic muscles, weakness, increased sensitivity to dTc, and detectable anti-nAChR antibody in serum. Our observation should be considered in the context of the proposed association between chronic infections and MG. The presence of anti-nAChR antibody in our model may explain the difference between our results and those of previous investigators. In addition, it is in itself of great interest, given that in human MG the causes of the autoimmune response are unknown. However, considerable problems exist with our analytic procedures, especially with regard to the high degree of nonspecific [I25I]α-bungarotoxin binding. Further careful studies with reliable methods are needed to confirm these results. In addition, because our various data were obtained from separate groups of animals, relationships between the various parameters studied, and thus a role of anti-nAChR antibody in impairment of neuromuscular transmission were not addressed here. Further study is required to define relationships between these variables and to better understand the molecular causes underlying antibody-nAChR interaction. Additionally, the duration of alterations in neuromuscular transmission after CLP injury needs to be determined to help pinpoint the specific effect of CLP injury on muscle.

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