Adverse effects of myasthenia gravis on rat phrenic diaphragm contractile performance

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Van Lunteren, Erik, Michelle Moyer, and Henry J. Kaminski. Adverse effects of myasthenia gravis on rat phrenic diaphragm contractile performance. J Appl Physiol 97: 895–901, 2004. First published April 23, 2004; 10.1152/japplphysiol.01266.2003.—Myasthenia gravis has variable effects on the respiratory system, ranging from no abnormalities to life-threatening respiratory failure. Studies characterized diaphragm muscle contractile performance in rat autoimmune myasthenia gravis. Rats received monoclonal antibody that recognizes acetylcholine receptor determinants (or inactive antibody); 3 days later, phrenic nerve and diaphragm were studied in vitro. Myasthenic rats segregated into two groups, those with normal vs. impaired limb muscle function when tested in intact animals (“mild” and “severe” myasthenic). Baseline diaphragm twitch force was reduced for both severe (P < 0.01) and mild (P < 0.05) myasthenic compared with control animals (twitch force: normal 1,352 ± 140, mild myasthenic 672 ± 99, severe myasthenic 687 ± 74 g/cm²). However, only severe myasthenic diaphragm had impaired diaphragm endurance, based on significantly (P < 0.05) accelerated rate of peak force decline during the initial period of stimulation (0.02 ± 0.02, 0.03 ± 0.01, and 0.09 ± 0.01%/pulse for normal, mild myasthenic, and severe myasthenic, respectively, during continuous stimulation) and intratrain fatigue (up to 30.5 ± 7.4% intratrain force drop in severe myasthenic vs. none in normal and mild myasthenic, P < 0.01). Furthermore, compared with continuous stimulation, intermittent stimulation had a protective effect on force of severe myasthenic diaphragm (force after 2,000 pulses was 31.4 ± 2.0% of initial during intermittent stimulation vs. 13.0 ± 2.1% of initial during continuous stimulation, P < 0.01) but not on normal diaphragm. These data indicate that baseline force and fatigue may be affected to different extents by varying severity of myasthenia gravis and furthermore provide a mechanism by which alterations in breathing pattern may worsen respiratory muscle function in neuromuscular diseases.

METHODS

Studies were performed on 20 female Lewis rats (weight 146–233 g, age 10–13 wk). Lewis rats are particularly susceptible to experimental autoimmune myasthenia gravis (EAMG) and have been a standard rat breed used for EAMG studies. The EAMG used for the present study is a passive model, in that the injected antibody (McAb-3) has direct effects on the neuromuscular junction (18). This

The integrity of neurotransmission from the phrenic nerve to the diaphragm muscle during repetitive stimulation is determined by the rate of acetylcholine release and recycling and its relationship to the safety factor of neurotransmission. Adult mammalian neuromuscular junctions have a relatively high safety factor (32), so that considerable decreases in transmitter release need to occur before neurotransmission fails. Despite the protective safety factor, neurotransmission failure does occur under experimental conditions, especially during prolonged activation at higher frequencies. In rat phrenic nerve and diaphragm, estimates of the maximal contribution of neurotransmission failure to diaphragm fatigue range from 15 to >75% (1, 14, 16, 31), with greater values generally being noted during higher stimulation frequencies (16).

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contrasts with the active model of myasthenia gravis (17), in which acetylcholine receptors from a different species are injected and the recipient species generates antibodies against the foreign protein, which then cross-reacts to affect its own acetylcholine receptors. Female rats were used in the original Lennon and Lambert (18) study, and acetylcholine receptors from a different species are injected and the contrasts with the active model of myasthenia gravis (17), in which...
angle of 90° ("mild myasthenic," \(n = 6\)). The second group all had weakness, with a reduced motor score (28.2 ± 2.0 out of a possible 36 points) and an inability to stay on the textured mat when held at a steep angle (steepest angle averaged 62.0 ± 5.4°) ("severe myasthenic," \(n = 8\)); both indexes differed significantly from those of normal and mild myasthenic animals (tested with one-way ANOVA followed by Newman-Keuls test).

Values for the peak diaphragm force in response to twitch and 50-Hz stimulation of the phrenic nerve are presented in Table 1. They demonstrate impaired force in both myasthenic groups, even in mild myasthenic animals with clinically normal limb muscle function. Thus about half of the myasthenic rats had normal limb muscle performance when tested in vivo but reduced in vitro diaphragm force, whereas the other half had impairments in both limb and diaphragm force.

Force declined faster as a function of time during continuous stimulation than during intermittent stimulation for both normal and myasthenic muscle strips (Fig. 1). Because there was a 0.67-s recovery period every second during intermittent stimulation, there was a larger number of stimulations per second during continuous stimulation. When force was compared as a function of pulse number, the force of severe myasthenic diaphragm declined more during continuous stimulation than during intermittent stimulation and was significantly different after 2,000 pulses (Fig. 2). (The 2,000 pulses corresponds to 40 s of continuous stimulation or slightly less than 2 min of intermittent stimulation. Studies examined a total of 2 min of intermittent stimulation, which corresponds to 2,040 pulses; statistical analysis of data after 2,040 pulses was similar to that after 2,000 pulses.) A similar but more modest trend was noted for the mild myasthenic diaphragms, but this did not reach statistical significance. In addition, there was no significant difference in the force decline during intermittent and continuous stimulation after 2,000 pulses for normal muscle strips.

In the severe myasthenic group, force loss during both intermittent and continuous stimulation reached a plateau within the time frame of the repetitive stimulation protocol. (For the severe myasthenic data of Fig. 1, the 120-s point during intermittent stimulation and the 110-s point during continuous stimulation were each slightly higher than the

### Table 1. Effects of myasthenia gravis on diaphragm force normalized for cross-sectional area (in g/cm²) during twitch and 50-Hz stimulation of the phrenic nerve

<table>
<thead>
<tr>
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<th>Normal</th>
<th>Mild Myasthenic</th>
<th>Severe Myasthenic</th>
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<tbody>
<tr>
<td>Twitch force</td>
<td>1,352±140</td>
<td>672±99*</td>
<td>687±74*</td>
</tr>
<tr>
<td>50-Hz force</td>
<td>3,116±564</td>
<td>1,843±295*</td>
<td>1,227±196*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significant reductions from normal diaphragm. Force values did not differ significantly between mild and severe myasthenic diaphragms. Statistical testing used 1-way ANOVA followed by Newman-Keuls test.

![Fig. 1. Rate of diaphragm muscle force loss during phrenic nerve stimulation as a function of time in normal, mild myasthenic (Mild MG), and severe myasthenic (Severe MG) rats. Force loss is compared for continuous and intermittent stimulation, with the former consisting of nonstop 50-Hz stimulation and the latter consisting of repetitive 50-Hz pulse trains of 0.33-s duration applied once per second. Values are means ± SE. *Difference in force values between intermittent and continuous stimulation. Statistical testing used repeated-measures ANOVA followed by Newman-Keuls test.](http://jap.physiology.org/)

![Fig. 2. Diaphragm force after 2,000 pulses of either intermittent or continuous phrenic nerve stimulation (means ± SE) in normal, mild myasthenic, and severe myasthenic rats. *Continuous stimulation differed significantly from intermittent stimulation. Statistical testing used 2-way ANOVA followed by Newman-Keuls test.](http://jap.physiology.org/)
DISCUSSION

Effects of myasthenia gravis on short-term contractions. The effects of myasthenia gravis on respiratory muscle function during single short-term contractions is well described in human studies. For example, Ringqvist and Ringqvist (25) studied nine subjects with moderate to rather severe generalized disease and found that the maximum inspiratory pressure was reduced to 78% of predicted, whereas the maximum expiratory pressure was reduced to 55% of predicted. Mier-Jedrzejowicz et al. (20) studied 17 subjects who had mild to moderate generalized disease with breathlessness and found ~25% of the total duration of intermittent stimulation. A relatively long duration was chosen for the initial rate of force decline to minimize the influence of the transient early force increase seen with some of the stimulation paradigms. The initial rate of force loss during intermittent stimulation was significantly greater in the severe myasthenic animals than in both the normal and mild myasthenic rats (Fig. 3). Similar findings were noted for continuous stimulation when calculated over the same number of pulses (Fig. 3). These findings persisted even when the rate of force decline was quantified after excluding the initial short period of force increase (i.e., the first 10 s) in the mild myasthenic muscle and persisted when alternate numbers of pulses where chosen for statistical analysis (200, 400, and 600 pulses).

Intratrain fatigue during intermittent stimulation was examined by measuring the force at the end of the 330-ms-long train and expressing it as a percent of the maximum force generated in that same train (Fig. 4). Control muscle and mild myasthenic muscle strips maintained force throughout each train during the 2 min of stimulation, so that F330 was a constant 100%. However, severe myasthenic muscle strips did not maintain force as well during the course of each train. The F330 gradually decreased during the fatigue run and was significantly different from that of both the control and mild myasthenic rats (tested statistically with repeated-measures ANOVA followed by the Newman-Keuls test).

Fig. 3. Rate of initial diaphragm force loss in response to nerve stimulation is compared for normal, mild myasthenic, and severe myasthenic rats during intermittent and continuous stimulation (means ± SE). The rate of force loss was calculated by dividing the total force loss after 500 pulses by the number of pulses to determine force loss per pulse. *Severe myasthenic rats differed significantly from the other 2 groups. Statistical testing used 2-way ANOVA followed by Newman-Keuls test.

Fig. 4. Changes in intratrain diaphragm force loss during phrenic nerve stimulation in normal, mild myasthenic, and severe myasthenic rats; data are from intermittent stimulation protocols only. A: tracings from a normal and a severe MG diaphragm. Note that, for normal diaphragm, force is maintained during the course of the train. In contrast, for diaphragm from the severe myasthenic animal, there is a decline in force during the course of the train. Time calibration, 250 ms. B: mean data of intratrain force loss over time from all 3 groups of animals, as assessed during intermittent stimulation. Intratrain force is quantified by the force at the end of the 330-ms train relative to peak force during that train (F330). A value of 100% indicates no force loss within the train. Data are expressed as means ± SE. Statistical testing used repeated-measures ANOVA followed by Newman-Keuls test. For the entire data set, severe myasthenic muscle differed significantly from both control and mild myasthenic muscle when tested with ANOVA (P = 0.006). *Severe myasthenic rats differed significantly from the other 2 groups at specific time points.
that the vital capacity was 71% predicted, the maximum inspiratory pressure was 54% of predicted, and the maximum expiratory pressure was 52% of predicted. More recently, Keenan et al. (13) found reduced maximum inspiratory pressure (70% predicted) and maximum expiratory pressure (50% predicted) in 13 subjects with varying degrees of generalized myasthenia gravis, but no abnormalities in respiratory muscle function in four subjects with ocular involvement alone. In the present study, the diaphragm of both groups of myasthenic animals had considerable impairment of short-term force generation in response to phrenic nerve stimulation, with values ranging from 39 to 59% of control for twitch and 50-Hz stimulation. Of interest, however, is that diaphragm force was reduced considerably even in the mild myasthenic animals with the load was graded according the baseline inspiratory muscle function. However, endurance of four subjects with ocular-only myasthenia gravis was not altered significantly.

The present study found that diaphragm fatigue was affected differently in the two groups of myasthenic animals, even after we factored out the effects of variability in initial diaphragm force generating capacity in the two myasthenic compared with the normal animal groups. First, the rate of force decline near the onset of stimulation was much faster in severe myasthenic than in normal neuromuscular junctions, whereas mild myasthenic diaphragm did not differ from normal. Second, severe myasthenic diaphragm exhibited intratrain force loss, which was not present in either mild myasthenic or normal preparations. Thus in the rat model of EAMG there is a complex relationship between alterations in limb muscle function tested during short-term contractions in vivo, diaphragm force tested during single contractions in vitro, and diaphragm fatigue tested over the course of several minutes in vitro.

Whether the pattern of stimulation was intermittent or continuous impacted force loss for all three groups, but to a greater extent for the severe myasthenic than for the normal phrenic diaphragm neuromuscular junctions. For the normal junctions, continuous stimulation was associated with a greater force loss as a function of time, but not as a function of pulse number (at least up to 2,000 pulses). In contrast, in the severe myasthenic junctions, force loss was greater with continuous than intermittent stimulation as a function of both time and number of pulses. There was a trend toward a similar finding in the mild myasthenic junctions. Thus the protective effect of intermittent stimulation on end-plate potential size (21, 22) appears to be much more important from the perspective of muscle contractile properties compared with healthy neuromuscular junctions.

Possible influence of muscle factors. The above discussion has focused on neuromuscular junction factors in determining force loss in the phrenic nerve-diaphragm muscle preparation. One needs to also consider that some of the findings of the present study were due to, or at least modulated by, alterations in muscle contractile and fatigue properties as opposed to purely neuromuscular junction changes. One issue is that muscle fiber properties could have been altered in the myasthenic diaphragm, not as a direct result of the disease, but potentially as a secondary consequence of altered diaphragm activation patterns. An advantage of the specific myasthenia gravis model used for the present studies is the rapid onset of the neuromuscular junction dysfunction, so that any secondary alterations in muscle fiber properties are unlikely to have been fully developed at the time the animals were studied. Nonetheless, early changes at 3 days may have been sufficient to produce some of the changes noted in the present study. A second issue is based on the possibility that neuromuscular junctions of different fiber types are differentially susceptible to failure. Thus the remaining population of fibers with functioning neuromuscular junctions may be comprised of a different distribution than found in the normal muscle, thereby contributing to alterations in force loss during repetitive stimulation. In normal muscle, fast fibers (in particular type IIB fibers) are more susceptible than slow fibers to neurotransmission failure because of a lower safety factor (4, 12, 23, 28). This was not tested on a single fiber basis in the present study and hence will require direct assessment in a future investigation.

Methodological issues. There is no generally accepted objective scale to evaluate weakness in animals with experimentally induced myasthenia gravis, and none has been fully validated in the strictest sense. The motor score chosen for the present study has been used in experimental spinal cord injury and was chosen on the basis of the use of multiple measurements to produce a composite score. Although the motor score used in the present study is somewhat subjective, many EAMG studies have used a simpler yet equally subjective scoring system dividing animals into mild, moderate, and severe categories (24, 30). Some studies have used “sensitization” tests to confirm the presence of a neuromuscular junction defect having been induced but do not provide an assessment of functional weakness in the intact animal. Injection of pachyromonium and edrophonium would have complicated contractility studies because of the need for a washout period before animal death, and there are no pharmacological data on the half-life of these agents in rats. Because we were using a well-accepted animal model and monoclonal antibodies that have been used to induce EAMG in other studies, we saw no reason to perform sensitization tests. Although there is an element of subjectivity in the motor score and incline plane tests chosen for the present study, there was a clear discriminatory threshold between the normal and mild myasthenic animals, on the one hand, and the severe myasthenic animals, on the other hand. That is, for the incline plane test, all of the normal and mild myasthenic animals made it to 90°, whereas none of the severe myasthenics did or even came close. Similarly, for the motor scale test, all of the normal and mild myasthenic animals were fully normal, whereas the severe myasthenic animals had multiple
abnormalities. Thus there was not an arbitrary value on the tests, below which the animals were placed in one group and above which they were placed in another.

It was unexpected at the onset of the study that the animals would segregate into two groups. This observation was exploited, thereby gaining further insight into diaphragm contractile performance as a result of this unexpected finding. The differences among animals in the severity of myasthenia contrast with the original report of this model (18), in which eight of eight animals were found to have muscle weakness. One major difference between the present study and the original description was in the route of antibody administration, being intraperitoneal vs. intravenous, respectively. The intraperitoneal route was based on Ruff and Lennon (27) as well as a desire to minimize animal stress and discomfort. Regarding the time at which the animals were studied, it does not appear that the choice of 3 days influenced the segregation of myasthenic animals into two groups. First, the original study of Lennon and Lambert (18) reported data 2–3 days after injection of the monoclonal antibody, so that the time frame of the present study was in the range of the original study. Second, on general inspection of the animals at 1 and 2 days after injection (done for health monitoring as part of the animal care protocol), it was easily discernable that animals injected with the active antibody (McAb-3) varied in severity of disease, with some animals clearly weak and others appearing totally normal (although this was not quantified with formal testing). Thus the difference was not primarily a function of some animals improving.

In summary, phrenic nerve-diaphragm function in the rat experimental autoimmune model of myasthenia gravis demonstrates many features in common with respiratory muscle dysfunction in human myasthenia. These include reductions in force during short-term contractions, as well as heightened rate of force loss in response to repetitive stimulation. In rats as in humans, there is variability in the extent of respiratory muscle dysfunction, and apparent variability in disease expression in respiratory compared with nonrespiratory skeletal muscles. The differential susceptibility of muscle groups could be related to differential populations of fiber types among muscles as well as differences in the frequencies at which various muscles are activated. The rat myasthenia model allows a close examination of the contractile function of the diaphragm in response to phrenic nerve stimulation, and this can be extended to studies of neuromuscular junction structural and biochemical properties without the immense difficulty of obtaining proper tissue from humans. Regarding the issue of whether the protective effect of intermittent stimulation on end-plate potential size is important from the perspective of muscle contraction, this appears to be more the case in diseased than healthy neuromuscular junctions.

These findings are significant in several respects. First, they point out that the severity of myasthenia gravis (as assessed by limb muscle testing) affects diaphragm contractile performance. That is, although both mild and severe myasthenia gravis impaired diaphragm muscle baseline force to an almost equal extent, severe myasthenia had a much greater effect on diaphragm fatigue. From a pathophysiological perspective, this provides evidence for a greater heterogeneity in phrenic diaphragm neuromuscular junction responses to myasthenia gravis that had been previously appreciated (previous notions had been that force generation and fatigue resistance decline in parallel). From a clinical perspective, the implication is that, despite equal degrees of diaphragm force loss when tested during a single effort, there may be subsets of patients with variable risk of developing respiratory failure when ventilatory demands are increased. However, respiratory system studies (e.g., breathing parameters such as tidal volume, respiratory rate, arterial blood gasses, and how they change in response to ventilatory loads) in intact rats with varying severities of myasthenia would better establish the link between the respiratory consequences of rat and human myasthenia. Second, the present findings point out key differences between normal and myasthenic muscle in force loss as a function of stimulation pattern. That is, in normal muscle, force loss appears to be directly related to the number of activations irrespective of whether the activation is continuous or intermittent (based on total force loss after 2,000 pulses). In contrast, in severe myasthenic muscle, force loss is dependent not only on the number of activations but also on the pattern with which these activations occur. Thus the brief opportunities for recovery that occur during the quiescent-phase intermittent activation are particularly important for myasthenic muscle. From a respiratory perspective, this suggests that alterations in breathing pattern that increase the duty cycle of diaphragm contraction (i.e., longer inspirations and/or shorter expirations, e.g., in response to chemical or mechanical perturbations or disease) may be especially deleterious for subjects with myasthenia gravis, thereby increasing their risk of developing respiratory failure and requiring mechanical ventilation. Physicians have observed differential involvement of muscle groups by myasthenia gravis for decades. The cellular and physiological bases are likely to be dependent not only on the extent of postsynaptic injury but also on properties on the presynaptic side. One obvious neuronal property that could influence the severity of neuromuscular transmission failure in myasthenia gravis would be the characteristics of the neuronal stimulation, which is the factor investigated in the present study.

GRANTS

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