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).

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).
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 females were also chosen for the present study so as to avoid introducing potential variability by also including male animals. The estrus cycle was not considered in the selection of animals, because sex hormones do not influence the severity of actively induced EAMG (17), and it would therefore seem less likely to influence the severity of passive-transfer-induced EAMG. All experiments were conducted in accordance with the animal care and welfare guidelines of the National Institutes of Health and were approved by the institutional animal care and use committee. Experimental rats were injected with monoclonal antibody solution consisting of 112.8 µl of stock McAb-3 (0.2 ml at a 0.1 nM concentration) (obtained from Dr. Vanda Lennon, Mayo Clinic, Rochester, MN), 84 µl of rat serum, and 643.2 µl of PBS to make a total of 840 µl ip injected per rat. McAb-3 recognizes rat acetylcholine receptor determinants and induces EAMG, which is clinically and pathologically similar to human myasthenia gravis (18, 19). In the original description of the McAb-3 monoclonal antibody study, animals were studied 2–3 days after injection and were found to have weakness, reduced miniature end-plate potentials, and reduced muscle twitch amplitudes (18). Control rats were injected with rat monoclonal antibody McAb-1 (also from Dr. Lennon), which recognizes determinants of the Torpedo, but not rat, acetylcholine receptor and does not induce EAMG in rats. The McAb-1 solution consisted of 50 µl of stock McAb-1, 84 µl of rat serum, and 706 µl of PBS for a total of 840 µl ip injected for each rat.

Three days after injection, rats were evaluated for severity of EAMG by a motor score (8) in which limb muscle deficit during locomotion, spreading of toes, and leg placement were rated so that a total score (out of a possible 36 points) was determined for each rat. Each rat was also given an inclined plane test (26) that tested its ability to grip a textured mat when placed on an inclined plane. The inclined plane test consisted of the following, described by Rivlin and Tator (26) but also used by Gale et al. (8). A ribbed rubber mat was cemented to a piece of wood, and this was placed at varying angles ranging from horizontal (0°) to vertical (90°). The rat was placed on the mat, and the incline was gradually increased until the animal could no longer maintain its position for 5 s. To avoid the complicating effects of motion on the animal, the animal was again placed on the mat at the angle at which it previously lost its position, and the angle was then adjusted as above. Final readings to the nearest 5° were taken from the stationary position. The motor score was based on that of Gale et al. It assessed three general things: 1) limb movement during spontaneous activity in an open field (rated from 0 to 5), 2) toe spread when the rats were picked up (rated from 0 to 2), and 3) foot placement on a hard edge when the animals were held off the ground (rated to 2). Specific measurements were as follows. Limb movement during spontaneous activity in an open field was rated from 0 to 5: 0 = no movement of limb and no weight bearing, 1 = barely perceptible movement of limb and no weight bearing, 2 = frequent and/or vigorous movement in limb but no weight bearing, 3 = can support weight on limb and may take one or two steps, 4 = walks only with mild deficit, 5 = normal walking. Toe spread was rated from 0 to 2: 0 = no spreading of toes, 1 = mild spreading of toes, 2 = normal full toe spread. Placing was rated from 0 to 2: 0 = no attempt to place foot, 1 = weak attempt to place foot, 2 = normal placing. All measures were done separately for each of four limbs in the present study because of myasthenia involving both fore- and hindlimbs, in contrast to Gale et al., who assessed only the hindlimbs as they were studying T8 spinal cord injury with isolated hindlimb dysfunction. Thus, in the present study, each limb could have a maximal score of 9 points, hence a score of 36 points for the whole animal indicates normal function. Gale et al. also tested withdrawal responses to extension, pain, and pressure, as well as righting when the animals were rolled on their backs. The withdrawal reflexes were not tested because of a desire to minimize discomfort to the animals, and the righting reflex was not tested because this does not give separate information about each of the four limbs.

Rats were subsequently anesthetized with urethane (initial dose 1 g/kg ip, with additional doses of 0.1–0.2 g/kg ip given as needed). The diaphragm and phrenic nerve were removed surgically. The muscles were placed in physiological solution comprised as follows (in mM): 135 NaCl, 5 KCl, 2.5 CaCl2, 1 MgSO4, 1 NaH2PO4, 15 NaHCO3, and 11 glucose with pH adjusted to 7.35–7.45 while being aerated with 95% O2–5% CO2. Small muscle ANOVA (1–1.5 mm diameter) were made, keeping the phrenic nerve as well as the rib and central tendinous insertion intact. Muscle strips were mounted vertically in an oxygenated double-jacketed bath. The temperature used for all contractile studies was 37°C, chosen over cooler temperatures because of its more physiological nature. The phrenic nerve was stimulated (pulse width 0.2 ms, supramaximal voltage) via a suction electrode (A-M Systems, Everett, WA), and the muscle was placed at optimal length. Isometric force was measured in grams with a high-sensitivity transducer (Kent Scientific/Radnoti Glass, Monrovia, CA). Diaphragm muscle strips were allowed to equilibrate and subsequently underwent twitch stimulation at 0.1 Hz for a baseline period of 3 min. Muscle strips were accepted for study only if twitch force varied by no more than 5% during the baseline period. Muscle strips then underwent either continuous or intermittent stimulation at a frequency of 50 Hz for a period of 2 min. In the rat, phrenic motoneurons have discharge rates between 34 and 76 Hz with a mean of 56 Hz during resting breathing (15), so that 50 Hz is near the middle of the normal range of activation frequencies. During intermittent stimulation, trains of pulses with a train duration of 0.33 s were delivered once per second (hence 17 pulses per second), thereby allowing the neuromuscular junction a 0.67-s recovery period before the onset of the subsequent train. Each muscle strip underwent only a single stimulation paradigm.

Muscle force records were digitized, collected online (Axotape, Axon Instruments, Foster City, CA), and stored on the hard drive of a computer for future analysis. Measurements of force were made with manually controlled cursors to report peak values. Values for force normalized for cross-sectional area in response to twitch and 50-Hz stimulation were calculated on the basis of the following formula (5): cross-sectional area × (muscle mass) × (fiber length)−1 × (muscle specific gravity)−1, assuming a muscle density of 1.06 g/ml. During fatigue testing, to factor out the effects of interstrip variability in size as well as myasthenia-induced baseline force reductions, all force values were normalized to the initial 50-Hz tetanic force measurement immediately preceding the fatigue test. Intratracheal fatigue was assessed by measuring the force at the end of the 330-ms-long train and expressing this as a percentage of the maximum force within the same tetanus (F330) (31).

All values presented are means ± SE. Statistical analysis used the unpaired t-test for comparison of two groups, as well as one-way, two-way, and repeated-measures ANOVA, followed by the Student-Newman-Keuls test when the ANOVA indicated significance, for comparison of multiple groups. A P value of <0.05 (two-tailed) indicated significance.

RESULTS

Rats that received inactive antibodies (McAb-1) (normal controls, n = 6) all had a normal motor score (36 out of a possible 36 points), and all were able to stay on the textured mat at an incline of 90°. The rats that received active antibodies (McAb-3) were segregated into two groups on the basis of functional testing of the intact animal. The first group did not have demonstrable weakness on motor ratings (score 36 for all animals), and all were able to stay on the textured mat at an
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>
<th></th>
<th>Normal</th>
<th>Mild Myasthenic</th>
<th>Severe Myasthenic</th>
</tr>
</thead>
<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. 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.

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.
The initial rate of force loss at the onset of stimulation was calculated over the first 500 pulses, which corresponded to ~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).

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
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 well-preserved limb muscle function (based on in vivo functional tests). These data therefore indicate that, in myasthenia gravis, diaphragm short-term force generation may be impaired substantially even in the absence of grossly apparent limb muscle dysfunction. The findings of the present study of in vitro diaphragm contractile performance need to be extended to assessments of diaphragm function and respiratory system performance to allow a more thorough comparison to human myasthenia gravis.

**Effects of myasthenia gravis on repetitive contractions.** We are aware of only limited studies examining respiratory muscle performance during repetitive activation in myasthenia. Keenan et al. (13) found that 13 subjects with varying degrees of generalized myasthenia had reduced diaphragm endurance on the basis of an incremental threshold loading test in which the load was graded according the baseline inspiratory muscle force. 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 intratraining 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 function in diseased 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 pancuronium 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.

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