The role of readthrough acetylcholinesterase in the pathophysiology of myasthenia gravis

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ABSTRACT Alternative splicing induces, under abnormal cholinergic neurotransmission, overproduction of the rare “readthrough” acetylcholinesterase variant AChE-R. We explored the pathophysiological relevance of this phenomenon in patients with myasthenia gravis (MG) and rats with experimental autoimmune MG (EAMG), neuromuscular junction diseases with depleted acetylcholine receptors. In MG and EAMG, we detected serum AChE-R accumulation. In EAMG, we alleviated electromyographic abnormalities by nanomolar doses of EN101, an antisense oligonucleotide that selectively lowers AChE-R in blood and muscle yet leaves unaffected the synaptic variant AChE-S. Whereas animals treated with placebo or conventional anticholinesterases continued to deteriorate, a 4 wk daily oral administration of EN101 improved survival, neuromuscular strength and clinical status in moribund EAMG rats. The efficacy of targeting only one AChE splicing variant highlights potential advantages of mRNA-targeted therapeutics for chronic cholinergic malfunctioning.—Brenner, T., Hamra-Amitay, Y., Evron, T., Boneva, N., Seidman, S., Soreq, H. The role of readthrough acetylcholinesterase in the pathophysiology of myasthenia gravis. FASEB J. 17, 214–222 (2003)

Key Words: neuromuscular function · MG · acetylcholine

Numerous impairments in neuromuscular function involve changes in the homeostasis of acetylcholine (ACh) and its destruction by acetylcholinesterase (AChE). Notable are syndromes of muscular origin such as muscular dystrophy (1) and motoneuron diseases like amyotrophic lateral sclerosis (ALS, ref 2), congenital myasthenias (3), and myasthenia gravis (MG), in which autoantibodies to the nicotinic ACh receptor (nAChR) induce neuromuscular junction (NMJ) malfunction (4).

Recent information indicates that nAChR and AChE functions in NMJs are intimately related. For example, compound mutagenesis of the ACHE gene and the gene for the α subunit of nAChR limits the severity of the impaired neuromuscular phenotype that is caused in zebrafish by ACHE disruption alone (5). Based on this and related information, we hypothesized that distortions in the AChE-nAChR balance may be relevant to several neuromuscular diseases. In the mammalian nerve and muscle, for example, imbalanced cholinergic neurotransmission induces enhanced transcription and shifted splicing of AChE pre-mRNA, leading to overproduction and accumulation of the normally rare readthrough AChE-R variant (6). In the immediate response to either acute stress or exposure to anticholinesterases, elevated AChE-R attenuates the initial hyperexcitation (7). However, continued accumulation of AChE-R may be detrimental, as it prolongs the state of cholinergic impairment (8, 9), increases adhesive and morphogenetic noncatalytic activities of AChE (10, 11), and is associated with muscle pathologies (12), predicting additional long-term effects under AChE-R overproduction. The synaptic AChE-S variant forms multimers that are attached to the membrane through the proline-rich PRiMA anchor (13). In contrast, AChE-R appears as soluble, secretory monomers that lack the carboxyl-terminal cysteine that is essential for linkage to PRiMA (14). Thus, each AChE variant is specialized: AChE-S for hydrolysis at the synapse and controlling its properties and AChE-R for nonsynaptic hydrolysis and morphogenesis. Consequently, the signaling action of ACh at the receptor, its cessation by hydrolysis, and the morphogenic activity of AChE may all be involved in the maintenance of neuromuscular functioning in a manner dependent on the composition of AChE variants.

MG of humans (4, 15) and experimental autoimmune MG (EAMG) of rats (16) present valuable systems for testing the involvement of AChE variants in the pathophysiology of NMJ diseases. Loss of receptors induces severe abnormalities in cholinergic neurotransmission, which cause neuromuscular weakness and accelerated, readily measurable muscle fatigue. Symptoms can be corrected by anticholinesterase therapy; however, therapy is effective for only short periods and chronic abnormalities in cholinergic neurotransmission persist. Our previous studies (8) (12) demonstrated AChE-R accumulation in muscle with impaired

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cholinergic neurotransmission. Therefore, we tested the accumulation of AChE-R in MG and EAMG and used variant-selective antisense agents to suppress AChE-R accumulation. Our findings demonstrate a role for AChE-R in EAMG pathophysiology and emphasize the advantage of targeting AChE-R mRNA in neuromuscular diseases that involve a shift in pre-mRNA splicing.

MATERIALS AND METHODS

Human MG patients

Serum samples from clinically identified MG patients were collected according to the guidelines and with the approval of the Hebrew University’s Bioethics Committee. The diagnosis of MG was based on positive titer of anti-AChR antibodies and typical clinical symptoms, including either a decremental response to repetitive nerve stimulation or a positive response to Tensilon test.

Materials

Unless otherwise specified, materials were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

EAMG was induced in female Lewis rats (120–150 g, Jackson Laboratory, Bar Harbor, ME) in accordance with NIH guidelines. FVB/N mice (Harlan Biotech Rehovot, Israel) and transgenic FVB/N mice that overexpress human (h)AChE-R lines. FVB/N mice (Harlan Biotech Rehovot, Israel) and Laboratory, Bar Harbor, ME) in accordance with NIH guidelines was determined using 125I-AChR concentration in the gastrocnemius and tibialis muscle.

Oligonucleotides

Lyophilized, HPLC-purified, GLP grade oligodeoxynucleotides (purity >90% as verified by capillary electrophoresis; Hybrion, Inc., Worcester, MA) were resuspended in sterile double distilled water (24 mg/mL) and stored at −20°C. Their sequences were:

- EN101 5’-CTGCCGATATTTCTTTGTA*G*C*-3’;
- EN102 5’-GGAGAAGAGGAGGAAGA*G*G*-3’; and
- invEN102 5’-GGAGAAGAGGAGGAAGA*G*G*-3’.

The three 3’-terminal residues (*) were substituted with oxymethyl groups at the 2’ positions. The EN101 and EN102 antisense oligonucleotides (AS-ONs) are complementary to the coding sequence of the rat AChE mRNA sequence (GeneBank accession no. S50879) common to all variants (18). The third is inverse (inv) to EN102 (19), and served as a negative control.

Antibodies

Rabbit polyclonal Abs against the carboxyl-terminal sequence of human AChE-R have been described (17). Goat polyclonal anti-AChR (S.C. 1148) was from Santa Cruz Biotecnology (Santa Cruz, CA). Biotinylated donkey anti-rabbit Ab (Chemicon International, Temecula, CA) and biotinylated donkey anti-goat Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary Abs.

Induction of EAMG

Torpedo ACh receptor (T-AChR) purified from T. californica electroplax on neurotoxin-Sepharose resin (20) was subcutaneously injected in the hind footpads of rats (40 µg T-AChR and 1 mg of M. tuberculosis H37Ra, Difco, Detroit MI, emulsified in complete Freund’s adjuvant). Booster injection of the same amount was administered after 30 days. Animals that did not develop EAMG after the second injection received a third one. Animals were weighed and inspected weekly during the first month, and daily after the booster immunization to evaluate muscle weakness. Their clinical status was graded as follows: (0) no weakness or fatigue, treadmill running time, 23 ± 3 min; (1) mildly decreased activity, weak grip with fatigue, weight loss >3% of body weight during a week, > 10 min running time on treadmill; (2) moderate weakness accompanied by weak grip, weight loss of 5–10%, 5–5 min running on treadmill; (3) moderate-severe weakness, hunched back posture at rest, head down and forelimb digit flexed, tremulous ambulation, 10% body weight loss, 1–2 min run on treadmill; and (4) severe general weakness, no audible complaint or grip, treadmill running time <1 min, weight loss >10%.

Serum analyses

Nondenaturing gel and catalytic activity measurements of AChE were as described (7). Iso-OMPA (tetraisopropylpyrophosphoramide) was used to block butyrylcholinesterase activity in serum samples (5×10−6 M) and polyacrylamide gels (5×10−6 M).

Anti-AChR Ab determination

Sera from EAMG animals and MG patients were assayed by direct radioimmunoassay for anti-Torpedo (T)-AChR, rat (R)-AChR, or human (H)-AChR (16, 20). All the EAMG rats displayed high anti-T-AChR and/or anti-R-AChR levels, with serum mean ± sd values of 82.1 ± 16.0 nM for anti-T-AChR Abs and 19.9 ± 1.8 nM for anti-R-AChR. MG patient sera were assayed by the same radioimmunoassay using H-AChR as antigen and displayed 1–60 nM Ab titers.

Quantification of nAChR

AChR concentration in the gastrocnemius and tibialis muscles was determined using 125I-α-bungarotoxin binding, followed by precipitation in saturated ammonium sulfate as described (21).

In situ hybridization

In situ hybridization was performed with fully 2’-O-methylated AChE-R or AChE-S-specific 50-mer cRNA probes complementary to AChE pseudointron 4 or exon 6, respectively (9). Detection was with alkaline phosphatase and Fast Red™ substrate (Molecular Probes, Eugene, OR). DAPI (Hoechst) counterstaining served to visualize nuclei.

Immunohistochemistry

Immunohistochemistry was performed on 7 µm paraffin-embedded muscle sections as described elsewhere (22). Primary Abs were diluted 1:100 and 1:30 for detecting rabbit anti-AChE-R and goat anti-AChR, respectively.

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Drug administration

Intravenous (i.v.) injection and blood sampling for anti-
AChR Ab determination were via the jugular vein under anesthesia. For oral administration, a curved intubation feeding
needle with a ball end (Stoelting, Wood Dale, IL) was used. Pyridostigmine (Mestinon Bromide™, Hoffmann-La Roche, Basel, Switzerland) was administered in a dose of 1 mg/kg per treatment, the highest dose that does not cause cholinergic crisis.

Electromyography

Rats were anesthetized by i.p. injection of 2.5 mg/kg pento-
barbital, immobilized, and subjected to repetitive sciatic nerve stimulation at 3 Hz, using a pair of concentric needle electrodes. Baseline compound muscle action potentials (CMAPs) were recorded by electrodes placed in the gastro-
neumius muscle following a train of repetitive nerve stimula-
tions at supramaximal intensity. Normal muscle shows no
decrease in action potentials. Decreases (percent) in the
amplitude of the decrease in action potentials. Decreases (percent) in the
amplitude of the CMAP ratio at supramaximal intensity. Normal muscle shows no
decrease in action potentials. Decreases (percent) in the
amplitude of the fifth vs. the first muscle action potential were
determined in two sets of repetitive stimulations for each
animal. A reduction of 10% or more indicated neuromuscu-
lar dysfunction (16). To assess the efficacy of the treatment,
the reduced CMAP in each animal was taken as a baseline and
changes were presented as comparison to this value. Thus, an
improvement of baseline decrement of 0.87 to 1.0 would yield
a value of the CMAP ratio of 115%.

Exercise training on treadmill

Animals were placed on an electrically powered treadmill (23) running at a rate of 25 m/min, an effort of moderate
physical intensity, until visibly fatigued. The time rats were
able to run was recorded before and after AS-ON or pyri-
dostigmine treatment.

RESULTS

AChE-R in blood of human MG subjects and EAMG
rats

AChE-R overproduction in MG was tested by separating
sera from mice and MG patients on nondenaturing polyacrylamide gels and staining for AChE activity. The
murine (m) monomeric AChE-R variant migrates on
such gels faster than the tetrameric synaptic enzyme
AChE-S (7). Serum from FVB/N mice subjected to
forced swim stress (9) displayed higher levels than
sera from transgenic mice, which overexpress human
(h)AChE-R (17), displayed an even more rapidly mi-
grating band, possibly due to the differences in the
carboxyl-terminal sequences between human and
mouse AChE-R or because of changes in the glycosyla-
tion of these two enzymes. Figure 1A presents these
iso-OMPA-resistant activity patterns. Similarly, fast-mi-
grating AChE was observed in the sera of 10 of 19 MG
patients whereas sera from 3 patients with an unrelated
disease (e.g., hepatitis) or from 2 healthy nonstressed
humans showed primarily a slower migrating AChE
form. Figure 1A further demonstrates this fast-migrat-
ing AChE variant in sera from 2 of 4 MG patients.
There was no apparent correlation between the inten-
sity of staining in the gels and anti-AChR Ab titers of the
analyzed patients (an average ± se of 22 ± 16 nM vs. 1.0
nM in healthy subjects). There was no correlation with
total serum AChE activity, which, on average, was
unchanged from levels in normal subjects. These ob-
servations suggested that the faster migrating bands
represent murine and human AChE-R (mR and hR,
respectively) and that AChE-R is over-represented in
the blood of some MG patients. A different method to
detect AChE-R in serum is by immunoblot analysis. In
the serum of EAMG vs. healthy rats, there was a massive
increase in immunopositive rat AChE-R (rR) that comi-
grated with hAChE-R (Fig. 1B).

AChE-R and AChE-R mRNA accumulate in muscles
of EAMG rats

Expression of alternative AChE variants (Fig. 2A) was
tested in control and EAMG rats. In a quantitative
binding of 125I-α-bungarotoxin, muscle nAChR was
reduced by 48 ± 7% from normal values in 10 mildly
affected rats (disease grade 1–2) and by 75 ± 5% in 10 severely affected rats (grade 4) compared with controls. Immunohistochemistry with antibodies to nAChR visualized this depletion in muscle sections from EAMG rats compared with control rats (Fig. 2B1, 2). Immunohistochemical staining with a polyclonal antiserum that selectively detects AChE-R (17) revealed positive signals in some but not all muscle fibers of control rats. Similar patterns appeared under treatment with the inert, inversely oriented oligonucleotide invEN102 (9; see Fig. 2B3 for invEN102-treated control muscle). In EAMG rats (Fig. 2B4), the same staining procedure showed dispersed cytoplasmic localization that is characteristic of this isoform, which is also secreted (6) and contrasts with the membrane-associated clusters of the synaptic variant (24). The level of expression and cellular distribution of muscle AChE-S were similar in EAMG and control, untreated, and invEN102-treated rats (data not shown).

Using in situ hybridization with variant-selective probes, we observed similarly distributed AChE-S mRNA in muscles from untreated and invEN102-treated healthy and EAMG rats (see Fig. 2B5, 6 for invEN102 treated muscle). In contrast, normal rats displayed only weak and diffuse labeling of the AChE-R mRNA transcript whereas pronounced punctuate labeling of AChE-R mRNA accumulations appeared in triceps muscles of EAMG rats, unaffected by invEN102 treatment (Fig. 2B7, 8). This accumulation indicated a selective overexpression of AChE-R in muscles of EAMG rats.

### AChE-R and AChE-R mRNA levels in muscle respond to EN101

The soluble, secretory nature of AChE-R predicted it would degrade ACh before it reached the postsynaptic membrane, limiting receptor activation. To test this hypothesis, we used the EN101 AS-ON, which is capable of selective suppression of AChE-R production (9, 25). Rat EN101 efficacy was tested in healthy and EAMG rats. EAMG muscle presented reduced nAChR staining (Fig. 2C1, 2). One day after a single i.v. injection of 250 μg/kg EN101, immunohistochemical staining demonstrated that AChE-R was significantly reduced in muscles from control and EAMG rats (Fig. 2C3, 4). In contrast, AChE-S remained essentially unchanged (data not shown). Under rEN101 treatment, receptor labeling intensity remained high in healthy rats and low in EAMG animals, similar to untreated animals and animals treated with invEN102 (compare Fig. 2B1, 2 with Fig. 2C1, 2). In situ hybridization indicated that AChE-S mRNA labeling was only nominally affected by EN101, suggesting that neuromuscular transmission would be maintained under this treatment (Fig. 2C5, 6). In contrast, EN101 reduced AChE-R mRNA labeling almost to the limit of detection in control and myasthenic rats (Fig. 2C7, 8).

### Suppression of AChE-R restores normal CMAP ratios in EAMG rats

Immunoblot analysis followed by densitometric quantification confirmed the increase in serum AChE-R in EAMG. A single i.v. injection of 250 μg/kg EN101, but not invEN102, reduced the serum level of AChE-R 24 h later in control and EAMG animals (Fig. 3A). To evaluate the physiological outcome of this suppression, we recorded CMAPs from the gastrocnemius muscle. EAMG rats, but never control animals, displayed a decrement in CMAP during repeated stimulation at 3 Hz. The baseline decrement, the percent of difference in the heights of the fifth and the first evoked potentials, ranged from 10% to 36% (mean±s.e.=13.0±2.5%,
Fig. 3B) compared with 4.0 ± 0.9% in healthy controls. Standard therapy for MG is administration of an anticholinesterase that elevates ACh levels to a threshold enabling receptor activation. Accordingly, we administered i.p. neostigmine bromide (Prostigmine™, Hoffmann-La Roche, 75 μg/kg). This rapidly corrected the CMAP decrement in EAMG rats from 87.6% of the first basal stimulation in untreated EAMG animals, which was considered as 100% baseline, to > 105% of the first evoked potential (i.e., 120% of the baseline). The effects of the cholinesterase blockade were evident starting 15 min after injection yet lasted only 2 h, after which time CMAP reverted to the decrement baseline (Fig. 3C).

Unlike anticholinesterases, which block all AChE variants, EN101 was shown to selectively suppress muscle AChE-R production (12). Therefore, retrieval of stable CMAP peaks in EN101-treated EAMG rats could attest to the causal role of AChE-R in myasthenic neuromuscular malfunctioning. Injection (i.v.) of EN101 at doses ranging from 50 to 500 μg/kg (2–20 nmol/rat) did not affect CMAP in control animals, but in EAMG rats retrieved stable, normalized CMAP ratios within 1 h (Fig. 3B, Fig. 4A, and Table 1). CMAP normalization was accompanied by increased mobility, upright posture, stronger grip, and reduced tremulousness of ambulation. The extent and the duration of CMAP correction were dose dependent; for example, 500 μg/kg conferred ≥ 72 h rectification of CMAP up to 125% of baseline whereas 50 μg/kg was effective for only 24 h. EN102, a similarly 3'-protected AS-ON targeting another sequence in AChE mRNA (AS1 in ref 19), produced similar rectification of CMAP decrements in EAMG rats, supporting the relevance of AChE-R as a contributing element to these decrements. Comparable amounts of invEN102, an inverted sequence with no target in the database, did not improve muscle function, attesting to the sequence specificity of the EN101 effect (Table 1). Dose response curves revealed that up to 5 h after an injection, EN101 produced a saturable response with an IC_{50} of <10 μg/kg. This effect appeared to be superimposed on a much longer lasting and less concentration-dependent effect that showed no saturation in the range we studied (Fig. 4B), possibly reflecting altered muscle and/or NMJ properties under the stable CMAP retrieval afforded by EN101.

Antisense prevention of AChE-R accumulation promotes stamina in EAMG rats

Placed on a treadmill at 25 m/min, control rats ran for 23.0 ± 3.0 min, then displayed visible signs of fatigue. Starting at 5 h and for at least 24 h after i.v. administration of 250 μg/kg EN101, EAMG rats demonstrated improved performance on the treadmill. Running time increased from 247 ± 35, 179 ± 21, and 32 ± 6 s to 488 ± 58, 500 ± 193, and 212 ± 59 s for animals at disease grades 2, 3, and 4, respectively (average values for 6–9 animals per group). Healthy animals, in contrast, were not significantly affected by EN101 injection.

2'-Oxymethyl protected AS-ON agents are efficient under oral administration (26). The dose of 50 μg/kg of EN101 was administered to EAMG rats once a day via an intubation feeding needle and CMAP measured 1, 5, and 24 h later. Orally administered 50 μg/kg EN101...
TABLE 1. Post-treatment CMAP ratios

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<th>Oral^a</th>
<th>Intravenous^b</th>
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<tr>
<td></td>
<td>Naive EN101</td>
<td>EAMG EN101</td>
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<tr>
<td>0 h</td>
<td>1.00 ± 0.00</td>
<td>0.87 ± 0.01</td>
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<td></td>
<td>(6)</td>
<td>(6)</td>
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<tr>
<td>1 h</td>
<td>1.0 ± 0.02</td>
<td>0.97 ± 0.02</td>
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<td></td>
<td>(4)</td>
<td>(8)</td>
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<tr>
<td>5 h</td>
<td>1.03 ± 0.02</td>
<td>0.97 ± 0.03</td>
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<td></td>
<td>(4)</td>
<td>(7)</td>
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<tr>
<td>24 h</td>
<td>1.01 ± 0.00</td>
<td>1.01 ± 0.01</td>
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<td></td>
<td>(7)</td>
<td>(6)</td>
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^a CMAP ratios (5th vs. 1st amplitude) were determined at the noted times after treatment and the averages ± SEM are presented. Each treatment represents similarly although not simultaneously treated rats, the numbers of which are shown in parentheses. The average initial CMAP ratio of each animal at 0 h was considered as 1.00. Thus, 0.84 reflects a 16% decrement in the pretreated EAMG animals, whereas 0.97 reflects a close to complete correction of their CMAP ratios by 1 h post-EN101 treatment.  

^b Drug doses were 50 μg/kg for EN101, EN102 or invEN102 and 1000 μg/kg for pyridostigmine for both administration routes.  

Note the apparently delayed effect of orally administered EN102 vs. EN101 or pyridostigmine.

and EN102 were as effective as 25 μg/kg EN101 administered i.v. (Table 1 and Fig. 4 and Fig. 5), but EN102 effects appeared somewhat delayed compared with EN101. Oral pyridostigmine (1000 μg/kg) restored CMAP for up to several hours whereas invEN102 had no significant effect (Table 1).

Long-term antisense AChE-R suppression modifies the course of EAMG pathophysiology

The long-term maintenance of stable CMAP under EN101 enabled us to test whether the cholinergic impairment contributes to the progressive physiological deterioration in EAMG. Rats were first treated with EN101 once a day for 5 days, CMAPs being determined before each treatment. The efficacy of EN101 and its capacity to reduce the inter-animal variability in CMAP values were comparable to those of pyridostigmine (Fig. 5A, B). However, the onset of response to pyridostigmine was more rapid (Table 1) whereas that observed with EN101 was longer lasting. Daily oral or i.v. administration of EN101 stabilized CMAPs over the entire course of treatment (Fig. 5B). In contrast, the effect of pyridostigmine wore off within several hours, causing pronounced fluctuations in muscle status (Table 1, Fig. 5).

Five of six animals with grade 3 severity of EAMG symptoms treated once daily with pyridostigmine died within 5 days. In contrast, six of eight animals with similar disease severity treated orally once daily with EN101 survived the full 5 day period. To exclude the possibility that this difference reflects the susceptibility of EAMG rats to the repeated anesthesia and CMAP measurements, we subjected groups of moderately sick animals to 1 month of daily oral treatment with minimal interference. EAMG rats receiving daily oral doses of EN101, presented significant improvement in survival, clinical status, and treadmill performance compared with pyridostigmine- and saline-treated animals (Fig. 6; P<0.041 for 4 wk survival incidence, Fisher exact test, AS-ON vs. other treatments). One-way repeated measures ANOVA yielded P<0.05 for all other measures (AS vs. other treatments at 4 wk). Rats in the saline- and pyridostigmine-treated groups lost weight (13.5 and 11 g/animal, respectively) whereas animals in the EN101-treated group gained weight, on average 13 g/animal, during the treatment period.

DISCUSSION

Our observations support the notion that AChE-R plays a role in MG pathophysiology and call for evaluation of the rationale of long-term mRNA-targeted therapy for imbalanced cholinergic function at NMJs.

Figure 5. Stable reversal of decremental CMAP response in EAMG rats treated orally with EN101. EAMG rats received EN101 once daily for up to 4 days by i.v. injection. CMAP ratios were determined in EAMG rats 1, 5, and 24 h after the first i.v. (25 μg/kg) or oral drug administration (50 μg/kg) of EN101 or pyridostigmine (1000 μg/kg), then every 24 h before the administration of the subsequent dose. A) Single dose. Orally administered pyridostigmine (n=4) relieved the decremental CMAP responses for 5 h. In contrast, EN101 treatment (n=8) relieved the decrement for 24 h. B) Repeated daily doses. Equivalent improvement is shown in muscle function after oral (50 μg/kg, n=8) compared with i.v. (25 μg/kg, n=4) administration of EN101.

ANTSENSE-BASED THERAPY OF MYASTHENIA GRAVIS
Cholinergic balance considerations

Neuromuscular homeostasis involves, among other elements, a balance between NMJ-bound AChE and AChR that regulates neuromuscular maintenance and functioning (3, 5). When this balance is impaired, notably in the cases of inherited or acquired MG and EAMG or under exposure to organophosphates (27), deterioration of muscle performance ensues. Figure 7 presents a scheme of the key elements controlling the cholinergic balance in NMJs and the putative mechanism through which it may be retrieved in MG and EAMG.

Pharmacological inhibition of AChE, a primary treatment of MG for many decades, assumed that AChE plays no role in the etiology or pathophysiology of MG; and that long-term use of AChE inhibitors does not contribute to MG or its symptoms. By damping the destruction of ACh by AChE, anti-cholinesterase therapy can transiently compensate for the reduced numbers of AChRs at NMJs (28). However, AChE-S blockade by the nondiscriminative anticholinesterases induces AChE-R overproduction (7). This would limit the duration of anticholinesterases’ capacity to retrieve stable CMAP and intensify the disease-associated cholinergic imbalance.

CMAP increases occurred under anticholinesterase treatment (for a few hours) and in EN101-treated animals (>24 h after treatment). The rapid onset of CMAP improvement under EN101 is most likely due to its easy accessibility to muscle tissue and a rapid turnover of its target mRNA. The long-lasting functioning of the AS-ON may indicate that there is no physiological change until AChE-R reaccumulates to a level at which it can cause NMJ malfunction. Under daily EN101 treatment, the steady reversal of the decremental change likely enabled sustained recovery processes, unlike the fluctuating values under conventional therapy. Recently, ACh was shown to suppress interleukin (IL) 1β production in macrophages (29, 30). The causal involvement of IL-1β in the myasthenic phenotype (31) suggested that the EN101 effect could be partially mediated by its persistent capacity to reduce AChE and therefore increase ACh and suppress IL-1β levels. Interrelationships between NMJ cholinergic imbalance and stress-induced change in the cytokine balance, recently reported to affect EAMG pathophysiology (31–33), may explain at least part of the continuously improving clinical scores under prolonged EN101 treatment.

Drug dose and time dependence

By directing nanomolar doses of an orally delivered AS-ON drug to the long-known MG target AChE, we achieved rapid yet long-lasting clinical improvement, associated with stable reversal of the CMAP decrement response at 3 Hz nerve stimulation and increased...
muscle stamina in the treadmill test. The beneficial effect of AS-ON injection on the CMAP response began within 1 h post-treatment and lasted many more hours than the effect of anticholinesterases, likely reflecting mechanistic differences between these two groups of drugs.

Chemical anticholinesterases stoichiometrically inhibit the large number of AChE-S molecules present in the NMJ (3000–5000 molecules/µm²; ref 34). By contrast, AChE mRNA chains exist in far lower quantities than their protein products (35). AChE-R mRNA normally is the least abundant of the alternative splice variants of AChE pre-mRNA (6). AS-ON agents that target the AChE-R mRNA transcripts can operate repeatedly, i.e., one AS-ON is responsible for hydrolysis of many mRNAs (36). This explains the > 100-fold difference in the molar dose of AS-ON vs. pyridostigmine that is effective in relieving EAMG weakness.

Because of the intrinsic instability of the AChE-R mRNA transcript (37), AS-ON agents targeted at the AChE mRNA sequence that is shared by all transcripts, such as EN101 or EN102, will destroy primarily the AChE mRNA sequence that is shared by all transcripts, mRNA transcript (37), AS-ON agents targeted at the terminal 2’/H11032 fi ciently prevented from being synthesized. The 3’ terminal 2’-oxymethyl protection of AS-ON chains increases the hybridization affinity while minimizing toxicity (25), explaining its efficacy after i.v. as well as oral administration. In comparison, conventional anticholinesterases would nonselectively inhibit the catalytic activity of the two variants and induce a robust, multi-tissue feedback response of AChE-R overproduction (7). This increases the noncatalytic activities of AChE (5) that are not necessarily blocked by anticholinesterases (5, 6). The long-term survival and improved clinical status and stamina of animals receiving AS-ON by daily oral treatment compared with the poor survival of severely diseased animals under the physiologically fluctuating effects of a single daily dose of placebo or pyridostigmine, highlight these differences.

Potential relevance to other diseases

The pathogenesis of MG and EAMG is primarily related to the destructive effect of anti-AChR Abs on the NMJ (4). Neuromuscular weakness associated with cholinergic imbalance is also known in patients with congenital myasthenic syndromes (3), where absence of the membrane anchor results in synaptic AChE-S being the only form missing, as well as after exposure to anticholinesterases, e.g., in Gulf War veterans (38). Other diseases with peripherally imbalanced cholinergic neurotransmission include muscle dystrophy (1), amyotrophic lateral sclerosis and post-traumatic stress disorder (2). As AS-ONS targeted to AChE mRNA provide relief of cholinergic muscle malfunction, they may be found useful for alleviating the weakness in these and other muscle malfunctions syndromes.

We dedicate this study to the memory of our longtime colleague and friend, Dr. Shlomo Seidman, who passed away during this study. This research was supported by grants from the Association Français contre les Myopathies (to T.B) and from the U.S. Army Medical Research and Materiel Command (DAMD 17–99–1–9547, to H.S.), Ester Neuroscience (to H.S. and T.B.), and the Eric Roland Center for Neurodegenerative Diseases of the Hebrew University. We thank Drs. M. Horowitz, I. Wirguin, Z. Argov, and D. Glick (Jerusalem) for helpful discussions.

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Received for publication July 18, 2002. Accepted for publication October 13, 2002.