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In vitro Selection of the 2'-Fluoro-2'-Deoxyribonucleotide Decoy RNA Inhibitor of Myasthenic Autoantibodies

SEO, HWA-SEON AND SEONG-WOOK LEE*

Department of Molecular Biology, Dankook University, Seoul 140-714, Korea

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Abstract Myasthenia gravis (MG) is caused mainly by autoantibodies directed against acetylcholine receptors located in the postsynaptic muscle cell membrane. Using *in vitro* selection techniques, we isolated an RNA containing 2'-fluoro pyrimidines that can specifically and avidly (K_d ~25 nM) bind rat monoclonal antibody called mAb198, which recognizes the main immunogenic region on the acetylcholine receptors. This RNA can act as a very effective decoy and block mAb198 binding to the receptors *in vitro*. Furthermore, this RNA decoy can prevent the antigenic modulation of the acetylcholine receptor caused by mAb198 in human muscle cell cultures with an IC_{50} of approximately 2.4 μM. These results indicate that the RNA selected in this study is a more potent decoy inhibitor of myasthenic antibodies than the previously identified RNA with 2'-amino pyrimidines [11]. Moreover, this RNA cross-reacts with autoantibodies from patients with MG and can protect human cells from the effects of these antibodies. These observations have important implications for developing an antigen-specific treatment of autoimmune diseases including MG, which is based on decoy RNAs selected *in vitro*.

Key words: *In vitro* selection, decoy RNA inhibitor, myasthenia gravis, acetylcholine receptor, autoantibody

Myasthenia gravis (MG) is a neuromuscular autoimmune disease associated with muscular weakness and fatigue. The pathogenesis of MG is mainly due to antibodies directed against acetylcholine receptors (AChR) located at neuromuscular junctions [3]. Animals immunized with purified AChR or passively transferred by anti-AChR antibodies have been reported to develop experimental autoimmune myasthenia gravis (EAMG) [14]. The majority of anti-AChR antibodies found in both human patients with MG and rats with EAMG recognize the main immunogenic region (MIR) in the extracellular domain of the AChR α-subunit [22, 23]. Such autoantibodies cause a reduction in the AChRs available on the skeletal muscle at neuromuscular junctions due to an accelerated internalization and degradation of the receptors, functional blockade of acetylcholine binding sites, and complement-mediated damage to the receptors [3, 14]. As a result, the neuromuscular transmission and muscular contraction is severely affected in MG patients. Because the current therapy for MG which includes general immunosuppression often results in toxic side effects [3], the development of a more improved therapy to specifically inhibit the autoimmune response to AChR is needed.

Since the symptoms of MG are mainly caused by autoantibodies, much effort has been directed toward the development of small molecules, such as anti-AChR Fab fragments or peptides, that bind these autoantibodies and block their interaction with AChRs [6, 16, 24]. However, the isolation of short peptides that avidly bind to target proteins has been difficult mainly due to their unstable folded structure [5]. In contrast, short RNA molecules can form a fairly stable tertiary structure via intramolecular base-pairing [4], and certain RNA molecules can function as specific decoy inhibitors of target proteins [8, 9]. Moreover, *in vitro* selection techniques can be employed to isolate short RNA molecules from random RNA libraries that bind with high affinity and specificity to several proteins including non-RNA binding proteins such as antibodies [2, 10, 21].

Previously, we isolated nuclease-resistant RNA decoys with 2'-amino-modified pyrimidines (2'-NH_2) that can bind and block a monoclonal antibody (mAb) 198 that is specific to the MIR present in human AChRs [11]. These RNAs are able to act as decoys and block the antibodies from their specific receptor, AChR. However, due to the relative low affinity of the selected RNA (SE RNA) and mAb198 (K_d=~60 nM) to that of the human AChR and the

*Corresponding author
Phone: 82-2-709-2905; Fax: 82-2-798-4733;
E-mail: SWL0208@unitel.co.kr

antibody (K_d=~22 nM), a high concentration of SE RNA is required to inhibit the activity of mAb198 in a cell culture [11]. This low affinity may be because the intramolecular helices containing 2'-amino-modified pyrimidines have a low melting temperature, thereby giving them thermodynamically unstable structures [17]. RNA molecules with a fluoro substitution at the 2' position of pyrimidines (2'-F) are also nuclease resistant [18] and can be synthesized by T7 RNA polymerase [1]. 2'-F RNAs selected against a specific target protein have a higher affinity than 2'-NH_2 RNAs, because 2'-F RNAs form substantially stronger intramolecular helices, thereby leading to more thermodynamically stable and more rigid secondary structures [17].

Accordingly, to develop RNA inhibitors of myasthenic autoantibodies with a higher affinity and more effective potency, we isolated an RNA decoy with 2'-fluoro pyrimidines that can specifically and avidly bind the monoclonal antibody, mAb198. This RNA was able to bind to the antibody more avidly and inhibit the antigenic-modulation of AChR on the cell surface more effectively than the RNA selected previously with 2'-amino pyrimidines [11]. In addition, this RNA cross-reacted with autoantibodies from patients with MG and acted as a potent decoy to inhibit the patients' autoantibodies to down-modulate AChR on human cells.

MATERIALS AND METHODS

Antibodies, AChR, and Cells

The rat mAb198 was kindly provided by Jon Lindstrom (University of Pennsylvania, Philadelphia, PA, U.S.A.). The serum samples from 3 patients with MG (batch #2051, #1; #2415, #2; and #1551, #3) were purchased from The Binding Site (San Diego, U.S.A.). The major extracellular domain of the human AChR α-subunit (amino acids 1-210) was a gift from Sohel Talib (Gencell, Santa Clara, U.S.A.). TE671 human medulloblastoma cell lines (ATCC 8805-CRL) of rhadomyosarcoma origin [20] express receptors for acetylcholine which is apparently identical to human AChR [15] and were used in the acetylcholine receptor down-regulation experiments.

Selection Procedure

A random pool of RNA oligonucleotides was generated by the *in vitro* transcription of synthetic DNA templates with 2'-deoxy-2'-fluoro CTP and UTP (Amersham, Arington Heights, U.S.A.) and normal GTP, ATP, and T7 RNA polymerase. The sequence of the resulting RNA library was 5'-GGGAGAGCGGAAGCGUGCUGGGCCN$_{40}$CA-UAACCCAGAGGUCGAUGG AUCCCCCC-3', where N_{40} represents 40 nucleotides (nts) with the equimolar incorporation of A, G, C, and U at each position. Ten micrograms of the RNA library were incubated with 5 μg of normal rat IgGs in 100 μl of a binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 1% BSA) for 30 min at room temperature with shaking. Thereafter, the antibody-RNA complexes were immunoprecipitated with 20 μl of protein G-Sepharose beads (Pharmacia Biotech, Piscataway, U.S.A.) and discarded to remove any RNAs that had bound to the constant region of the antibodies or adhered nonspecifically to the beads. The precleared supernatant was then transferred to a new tube and incubated with 2.5 μg of rat mAb198 for 30 min at room temperature. The mAb198-RNA complexes were immunoprecipitated and washed with 0.5 ml of the binding buffer. The RNAs were recovered and amplified as previously described [7]. The amplified DNA was used for 10 more rounds of selection, and the finally selected RNAs were analyzed as previously described [10, 11].

Analysis of selected RNAs

The selected RNA with 2'-deoxy-2'-fluoro pyrimidines (2'-F SE RNA) was radiolabeled and isolated as previously described [1, 10, 11]. The purified RNA was then incubated with different antibodies as described above. The antibody-RNA complexes were immunoprecipitated with protein G-Sepharose beads, and the bound RNAs were eluted from the pellets. The RNAs were analyzed on a 6% polyacrylamide gel with urea. For a gel shift analysis, the radiolabeled SE RNA was incubated with antibodies at room temperature for 30 min, and the complexes were analyzed on a 4% nondenaturing polyacrylamide gel.

Measuring Inhibition of mAb198-Binding to Human AChR by SE RNAs

The purified ectodomain of the AChR α-subunit (5 nM) was preincubated with 1 nM of ^{125}I-radiolabeled α-bungarotoxin (α-Bgt) in 100 μl of the binding buffer at 4°C for 3 h. MAb198 (10 nM) was then added in the presence or absence of competitor RNAs and incubated at room temperature for 1 h. The antibody-AChR complexes were immunoprecipitated with protein G-Sepharose beads and the bound radioactivity was measured. A sample without any antibody was used to determine the background level of ^{125}I α-Bgt found in the pellets. The extent of the inhibition was assessed, based on the decrease in the mAb198 binding to AChR in the presence and absence of the RNAs.

Assay of AChR Down-Modulation

The AChR down-modulation was monitored as previously described [11]. MAb198 (5 nM) or autoantibodies from patients with MG were preincubated in 90 μl of medium (DMEM) containing 10 mM $MgCl_2$, 1% BSA, and 10% FBS with the RNA library, and SE RNA, or no RNA for 30 min at room temperature. These mixtures were then added to TE671 cells in the presence of 40 μg/ml of cycloheximide and incubated at 37°C for 4 h. Next, 10 μl

of the medium containing 50 nM of ^{125}I-radiolabeled α-Bgt was added and incubated for another 3 h. The cells were then washed, and the bound α-Bgt was quantitated using a γ 5000 counter (Packard Instruments, Meridien, U.S.A.). The incubation of TE671 cells with 5 nM mAb198 by itself reduced the AChR expression on the cell surface by approximately 50%. This level of antigenic down-modulation was considered as 100%, and all other values were then normalized accordingly. For the antigenic down-modulation experiments with patient autoantibodies, patient sera were added to TE671 cells at a concentration which was determined to give similar levels of antigenic down-modulation as 5 nM mAb198: 1 μl of 1:2 dilution patient sera #1, 1 μl of 3:1 dilution of patient sera #2, or 1 μl of 3:5 dilution of patient sera #3 (data not shown).

RESULTS AND DISCUSSION

In Vitro Selection of 2'-Fluoro-2'-Deoxyribonucleotide RNA that Specifically and Avidly Binds to mAb198

An RNA library of approximately 10^{14} different molecules was created with every pyrimidine modified at its 2' position by a fluoro group. Each molecule in the library contained a 40-nucleotide (nt) long region derived from random sequences flanked by defined sequences. The RNAs bound to mAb198 were selected from the RNA library according to the method described in Materials and Methods. After 11 rounds of selection, the bound RNAs were amplified by RT-PCR reaction and cloned. Eleven different clones were then sequenced and were surprisingly found to contain the same sequence (Fig. 1A). More interestingly, the RNA sequence selected in this study with 2'-fluoro pyrimidines was exactly the same as that isolated previously with 2'-amino pyrimidines [11]. To determine if the selected RNA with 2'-fluoro pyrimidines (2'-F SE RNA) was bound specifically to mAb198, an immunoprecipitation experiment was performed using internally radiolabeled SE RNA (Fig. 1B). The SE RNA was shown to bind only mAb198 and not other rat IgGs (Fig. 1B, lanes 4-6). In contrast, the original library RNA with 2'-fluoro pyrimidines did not specifically bind to mAb198 (Fig. 1B, lanes 1-3). To confirm this binding specificity, a gel retardation experiment was also performed (Fig. 1C). The 2'-F SE RNA efficiently formed a shifted nucleoprotein complex with mAb198 (Fig. 1C, lane 2), but not with normal rat IgGs (Fig. 1C, lane 1). The formation of these antibody-RNA complexes was specifically inhibited only by the addition of a 10,000-fold excess of unlabeled 2'-F SE RNA, but not by a nonspecific competitor such as the library RNA (Fig. 1C, lanes 3 and 4).

The binding affinity of the SE RNA to mAb198 was determined by a gel retardation assay with trace amounts of radiolabeled RNAs and increasing amounts of mAb198

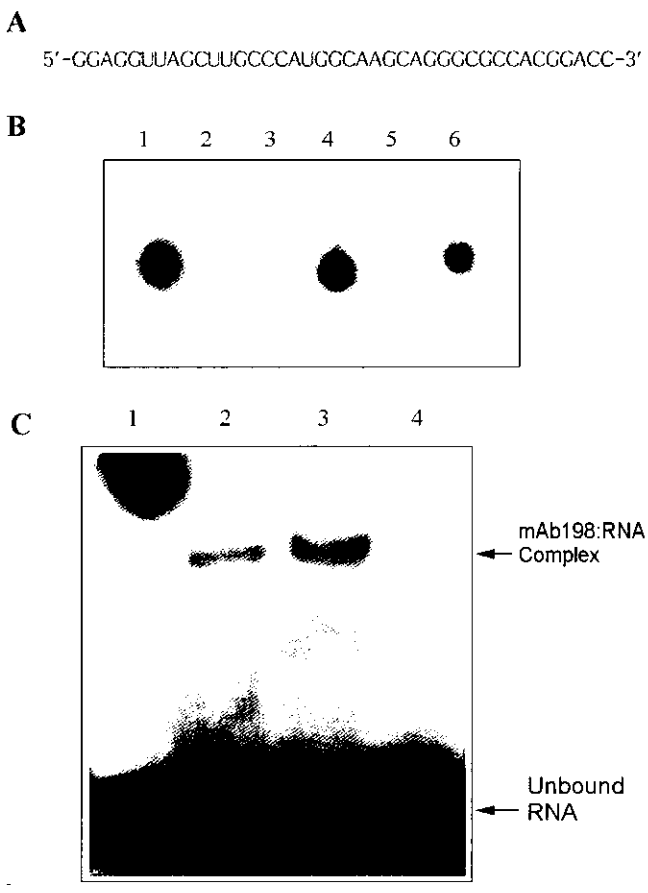

Fig. 1. Selected 2'-fluoro RNA (2'-F SE RNA) sequence and its specific binding to mAb198 antibody.
(A) Sequence of 2'-F SE RNA. (B) Specific binding of 2'-F SE RNA to mAb198. Radiolabeled 2'-F original library RNA (lanes 1-3, 1 nM) or 2'-F SE RNA (lanes 4-6, 1 nM) were incubated with normal rat IgG (lanes 2 and 5, 100 nM) or mAb198 (lanes 3 and 6, 100 nM). The antibody-RNA complexes were immunoprecipitated with protein G-Sepharose beads. The RNAs were then extracted and analyzed on a 6% polyacrylamide gel with urea. Lanes 1 and 4 contain 10% of the input RNA. (C) Specific binding of mAb198 to SE RNA. Radiolabeled 2'-F SE RNA (250 pM) was incubated with normal rat IgG (lane 1, 20 nM) or mAb198 (lanes 2-4, 20 nM). The original library RNA (lane 3, 2.5 μM unlabeled) or 2'-F SE RNA (lane 4, 2.5 μM unlabeled) were added in a 10,000-fold excess to the binding reaction as competitors to determine if either could specifically inhibit the formation of the mAb198-2'-F SE RNA complex. The resulting mAb198-RNA complexes were separated from the unbound RNA in a 4% nondenaturing acrylamide gel.

(Fig. 2). The equilibrium dissociation constant (K_d) was about 60 nM for the SE RNA selected previously with 2'-amino pyrimidines (2'-NH_2 SE RNA), as observed in previous experiments [11]. In contrast, a K_d of 25 nM, an affinity that was more than two-fold stronger than 2'-NH_2 SE RNA, was detected for 2'-F SE RNA. Hence, it would appear that 2'-F SE RNA binds very avidly to mAb198. In addition, it should be noted that the binding affinity of 2'-F SE RNA to mAb198 was very similar to the binding affinity of human AChR to the antibody (21.6±6.6 nM,

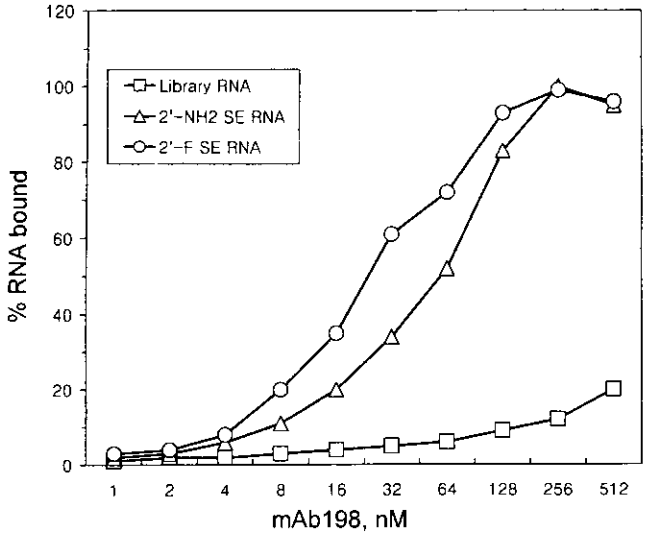

Fig. 2. Binding affinity between SE RNAs and mAb198.
The radiolabeled library RNA, 2'-NH_2 SE RNA (11), or 2'-F SE RNA isolated in this study (50 pM each) was incubated with increasing amounts of mAb198. The mAb198-RNA complexes were separated from the unbound RNAs on a 4% nondenaturing acrylamide gel. The percentage of RNA bound to mAb198 was calculated by determining the fraction of radioactivity present in the mAb198-RNA complexes. A maximum of 50% to 60% of the RNA was formed in the mAb198-RNA complexes, and the plotted numbers have been normalized to that amount.

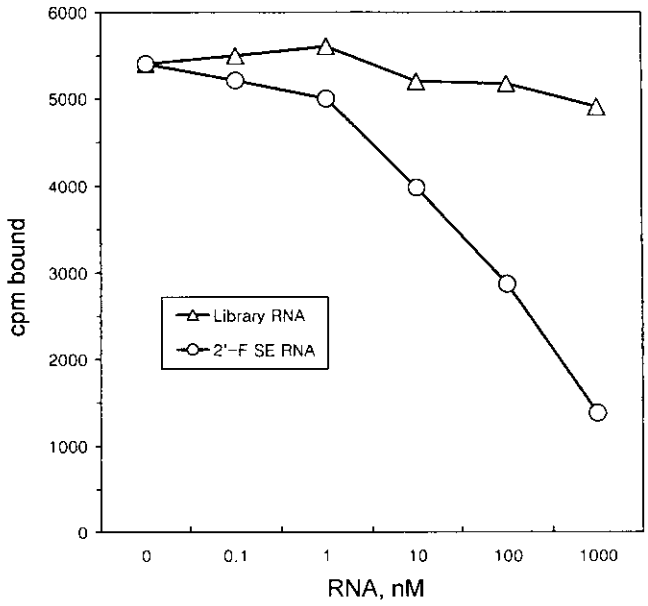

Fig. 3. Inhibition of mAb198 binding to human AChR by 2'-F SE RNA.
Human AChR was labeled with ^{125}I α-Bgt and incubated with mAb198 in the absence or presence of increasing amounts of library RNA or 2'-F SE RNA. The mAb198 bound to the receptor was quantitated after immunoprecipitation with protein G-Sepharose. The cpm numbers shown have been corrected by subtracting the background value of the radioactivity in the pellets without mAb198.

[16]). However, the original RNA library with 2'-fluoro pyrimidines was shown to have little affinity to mAb198, even in the highest concentration of the antibody.

2'-F SE RNA Blocks mAb198 Binding to and Down-Modulation of AChR on Human Cells

To determine if 2'-F SE RNA can specifically inhibit mAb198 binding to human AChR, the purified ectodomain of the human AChR α-subunit prebound to ^{125}I-radiolabeled α-Bgt was incubated with mAb198 in the presence or absence of a competitor RNA (Fig. 3). The purified human AChR α-subunit used in this study, which encompassed the major extracellular domain, was previously reported to induce EAMG in rats [13]. The incubated reaction complexes were immunoprecipitated and assayed for the ^{125}I α-Bgt content. The 2'-F SE RNA efficiently inhibited the interaction between mAb198 and human AChR in a concentration-dependent manner with an IC_{50} of 100 nM, while the original library RNA did not show any inhibition. Thus, 2'-F SE RNA is an effective decoy RNA, and most likely binds mAb198 at or near the antibody's combining site.

Once it was ascertained that 2'-F SE RNA avidly and specifically bound to mAb198 and that it could block mAb198 binding to its normal antigen (human AChR) in a test tube, the next step was to determine if this decoy RNA could protect cells from mAb198 in a culture. The incubation of human TE671 cells with mAb198 resulted in a dose- and time-dependent decrease in the number of AChR present on the cell surface (data not shown, [19]), which is referred to as antigenic down-modulation. To determine if 2'-F SE RNA could protect cells from the effects of mAb198 and inhibit the antibody-mediated down-modulation of AChR on human cells, TE671 cells were incubated with mAb198 in the presence or absence of the RNAs (Fig. 4). The degradation of the AChRs induced by the antibody was assessed by measuring the amount of ^{125}I α-Bgt that bound the AChRs which remained on the cells after treatment with the antibody, as previously described [11]. While a nonspecific RNA competitor, such as the original RNA library, did not inhibit the mAb198-mediated down-modulation of the AChR expression, the decoy 2'-F SE RNA protected the TE671 cells from mAb198 and inhibited the antigenic down-modulation by up to 75% (Figs. 4A and 4B). Furthermore, the 2'-F SE RNA-mediated inhibition of the antigenic down-modulation was dose-dependent with an IC_{50} of about 2.4 μM (Fig. 4B). Therefore, 2'-F SE RNA effectively protected cells from the mAb198-mediated down-modulation of AChR. Furthermore, the inhibition of the antigenic modulation by 2'-F SE RNA selected in this study was two-fold more efficient than that by 2'-NH_2 SE RNA observed previously (IC_{50}=~5 μM, 11), thereby indicating that the increase in the binding affinity of SE RNA to mAb198 improved the bioactivity of the RNA to inhibit the antibody effects. Indeed, we have previously shown that RRE decoy RNAs selected *in vitro*, which bind

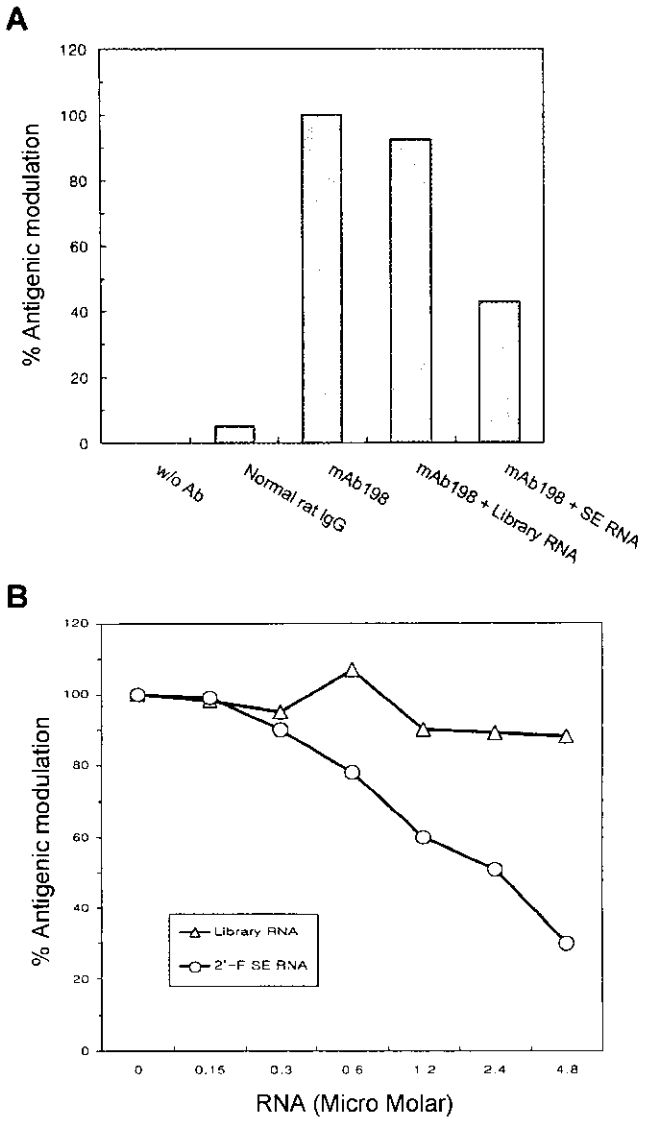

Fig. 4. Inhibition of mAb198-mediated antigenic down-modulation of AChR on TE671 cell surface by 2'-F SE RNA.

(A) TE671 cells were incubated with cell culture media containing no antibody (w/o Ab), normal rat IgG (5 nM), mAb198 (5 nM), mAb198 (5 nM) plus library RNA (3 μM), or mAb198 (5 nM) plus 2'-F SE RNA (3 μM). The down-modulation of the AChR expression was assayed by the ^{125}I α-Bgt binding to the cell surface AChRs. The values were expressed as a percentage of the down-modulation generated by mAb198 alone (5 nM) (% Antigenic modulation). (B) TE671 cells were incubated in culture media containing mAb198 (5 nM) along with increasing amounts of library RNA or 2'-F SE RNA, and the down-modulation of AChR was assayed as in (A).

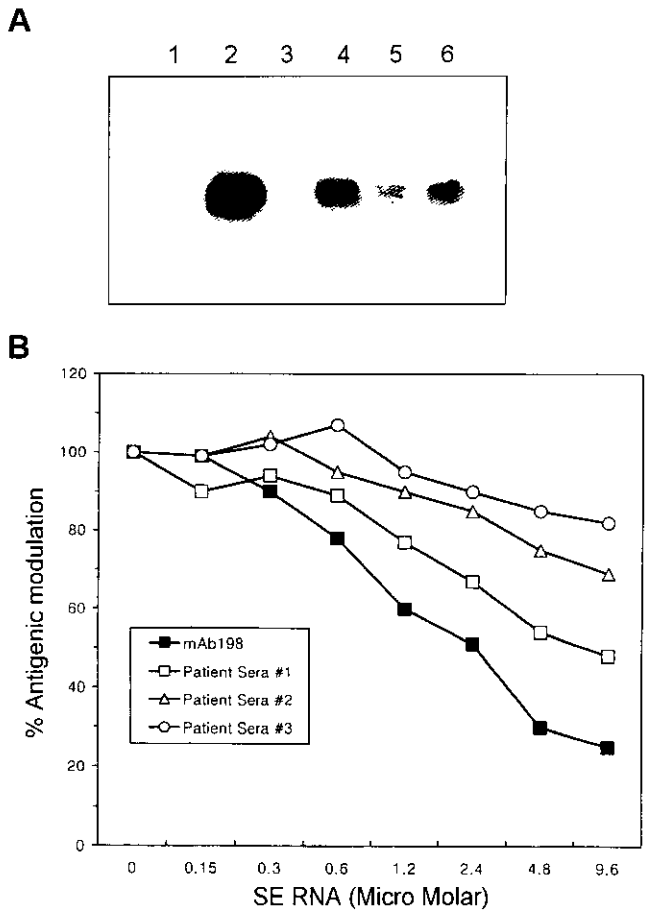

Fig. 5. Protection of cells from myasthenic autoantibodies by 2'-F SE RNA.

(A) 2'-F SE RNA was recognized by autoantibodies in sera from patients with MG. Radiolabeled 2'-F SE RNA (2.5 nM) was immunoprecipitated with normal rat IgG (lane 1, 100 nM), mAb198 (lane 2, 100 nM), normal human sera (lane 3, 50 μl), or three autoimmune patients sera (lane 4, sera #1; lane 5, sera #2; lane 6, sera #3; 50 μl each). The bound RNA was eluted and electrophoresed on a 6% polyacrylamide gel with urea. (B) 2'-F SE RNA inhibited the autoantibody-mediated down-modulation of AChR on the cell surface. TE671 cells were incubated in culture media containing mAb198 (5 nM), patient sera #1 (1 μl of 1:2 dilution), patient sera #2 (1 μl of 3:10 dilution), or patient sera #3 (1 μl of 3:5 dilution) that had been preincubated with increasing amounts of 2'-F SE RNA. The down-modulation of AChR was assayed by the I^{125} α-Bgt binding, as previously described. The values are expressed as the percentage of the down-modulation generated by mAb198 or patient sera alone.

to rev protein 10-fold better than wild-type RRE, are more potent inhibitors of HIV replication [12].

2'-F SE RNA Inhibits Autoantibody-Mediated Down-Modulation of AChR Expression

To explore the possibility that 2'-F SE RNA might be a structural mimicry of the main antigenic epitope of AChR that is recognized by the mAb198 antibody, and that the SE RNA would cross-react with patients' anti-AChR autoantibodies elicited by the MIR on the AChR, radiolabeled 2'-F SE RNA was incubated and immunoprecipitated with sera from 3 patients with MG (Fig. 5A). Patient sera with MG often contain autoantibodies that recognize the same MIR of human AChR as mAb198 [23]. As shown in Fig. 5A, lanes 4-6, all three of the autoimmune sera were found to contain antibodies that bound to the SE RNA, while the normal human serum did not (Fig. 5A, lane 3). Thus, 2'-F

SE RNA did appear to specifically cross-react with some fraction of the patients' autoantibodies.

These results would seem to suggest that 2'-F SE RNA resembles the common epitope of the MIR on AChR that is recognized by both mAb198 and MG autoantibodies. In addition, most pathologically relevant autoantibodies from MG patients appear to be bound to the MIR on AChR [14]. Therefore, SE RNA appears to be able to protect cells from a significant fraction of autoantibodies. To test this hypothesis, the ability of 2'-F SE RNA to protect cells from the three patients' autoimmune sera was evaluated (Fig. 5B). The incubation of TE671 cells with the patient sera was shown to cause a time- and dose-dependent antigenic down-modulation of AChR (data not shown, [11]). The addition of 2'-F SE RNA blocked a certain fraction of all the patient's autoantibodies from binding to and, hence, the down-modulation of AChR on TE671 cells in a dose-dependent manner. At the highest concentration of SE RNA tested, the down-modulation of AChR was inhibited by 52% with patient sera #1, 30% with #2, and 13% with #3. In particular, the SE RNA efficiently protected the cells from the autoantibody in the patient serum #1 with an IC_{50} of approximately 9 μM. Therefore, the 2'-F SE RNA was shown to cross-react with the autoantibodies in the sera from 3 patients with MG and protected a significant fraction of the AChRs present on the cells from the autoantibodies in the sera of at least one patient with MG. The different levels of SE RNA-mediated inhibition of the down-modulation detected in the sera from 3 patients may have been due to heterogeneous populations of anti-AChR autoantibodies in each patient serum [25], with some patients containing a higher fraction of autoantibodies against the MIR. Such heterogeneity of autoantibodies will limit the usefulness of the RNA selected in this study. However, the finding that a single decoy RNA can impede a significant fraction of a patient's autoantibodies from binding to their self-antigen is significant and suggests that one or a few selected decoy RNAs may be able to block the oligoclonal immune responses engendered by autoantibodies.

The pathogenic consequences of the autoimmune responses in patients with MG can be potentially modulated by molecules that bind to the combining site of autoantibodies and thus block their interaction with AChRs. To test this idea, we isolated a small, nuclease-resistant RNA molecule with 2'-fluoro pyrimidines that could specifically and avidly bind both a rat monoclonal antibody (mAb198) that recognizes the MIR on the AChR and autoantibodies from patients with MG. This RNA was found to be able to act as a decoy to block the antibody from binding to its natural antigens, AChRs, thereby protecting the human AChRs from the effects of these antibodies. Noticeably, the selection of RNA with 2'-fluoro groups increased the binding activity of the RNA to mAb198 and the potency of the RNA to inhibit the antibody activity by more than 2-fold, as compared to an SE RNA with 2'-amino groups. This may be due to the extreme thermostability and more rigid structure found in an RNA with 2'-fluoro groups, as shown in a previous report where 2'-F RNA ligands selected against a keratinocyte growth factor had higher affinities, bioactivities, and thermostabilities than 2'-NH_2 RNA ligands [17].

The observations in this study together with previous results [11] suggest that RNAs selected *in vitro* can be useful reagents to inhibit the autoantibody-mediated immune response against AChR in patients with MG. However, to be therapeutically valuable, more effective decoy RNAs that can block a larger fraction of autoantibodies need to be developed. This can be accomplished by selecting RNAs against additional anti-MIR monoclonal antibodies or various anti-AChR antibodies in MG patients. If a combination of such RNAs selected *in vitro* prevents the binding of a larger fraction of autoantibodies in a variety of MG patients, these RNAs can then be applied as lead compounds for the design of specific therapeutic agents against MG.

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