Prevention of passively transferred experimental autoimmune myasthenia gravis by a phage library-derived cyclic peptide

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Many pathogenic antibodies in myasthenia gravis (MG) and its animal model, experimental autoimmune MG (EAMG), are directed against the main immunogenic region (MIR) of the acetylcholine receptor (AcChoR). These antibodies are highly conformation dependent; hence, linear peptides derived from native receptor sequences are poor candidates for their immunoneutralization. We employed a phage-epitope library to identify peptide-mimotopes capable of preventing the pathogenicity of the anti-MIR mAb 198. We identified a 15-mer peptide (PMTLPENYFSERPYH) that binds specifically to mAb 198 and inhibits its binding to AcChoR. A 10-fold increase in the affinity of this peptide was achieved by incorporating flanking amino acid residues from the coat protein as present in the original phage library. This extended peptide (AEPMTLPENYFSERPYHPPPP) was constrained by the addition of cysteine residues on both ends of the peptide, thus generating a cyclic peptide that inhibited the binding of mAb 198 to AcChoR with a potency that is three orders of magnitude higher when compared with the parent library peptide. This cyclic peptide inhibited the *in vitro* binding of mAb 198 to AcChoR and prevented the antigenic modulation of AcChoR caused by mAb 198 in human muscle cell cultures. The cyclic peptide also reacted with several other anti-MIR mAbs and the sera of EAMG rats. In addition, this peptide blocked the ability of mAb 198 to passively transfer EAMG in rats. Further derivatization of the cyclic peptide may aid in the design of suitable synthetic mimotopes for modulation of MG.

Myasthenia gravis (MG) is a human neuromuscular disorder manifested by muscle weakness and fatigability of voluntary muscles. The symptoms of MG are mainly caused by an autoimmune attack on the muscle nicotinic acetylcholine receptor (AcChoR) located in the postsynaptic muscle cell membrane. Experimental autoimmune myasthenia gravis (EAMG) can be induced in animals by immunization with AcChoR or passively transferred by anti-AcChoR antibodies (1–3). Anti-AcChoR antibodies cause accelerated internalization and degradation of AcChoR by receptor crosslinking and complement-mediated lysis of the postsynaptic membrane, which cause AcChoR loss, failure of neuromuscular transmission, and paralysis (2, 4).

The antibody response to AcChoR in MG is heterogeneous. However, about two thirds of the antibodies formed, both in human MG and its experimental model, EAMG, are directed against the main immunogenic region (MIR), a small well-defined region in the extracellular domain of the AcChoR α-subunit (5). mAbs to MIR can passively transfer EAMG in rats and are capable of inducing AcChoR loss in muscle cell cultures (6–8). Because of the importance of MIR in pathogenicity of MG, much effort has been directed toward its characterization (5). By employing synthetic peptides and proteolytic fragments of the α-subunit of AcChoR, it has been shown that many anti-MIR antibodies bind to the peptide corresponding to residues 67–76 of the AcChoR α-subunit (5, 9, 10). However, it should be noted that these antibodies bind in a much weaker extent to the peptide than to the native receptor, thus indicating that other regions are present in close proximity to the MIR peptide in the intact native AcChoR and play a role in determining its antigenicity (2). Indeed, denaturation of AcChoR completely abolishes the activity of most anti-MIR mAbs. The limited utility of peptides corresponding to sequence determinants as immunoneutralizing agents for anti-MIR antibodies prompted us to search for synthetic mimetics of MIR by using phage-displayed random peptide libraries.

Phage display technology provides an unprecedented opportunity to select ligands for a variety of target proteins including antibodies (11–13). Earlier work in our laboratory has demonstrated the usefulness of phage-epitope libraries to select mimotopes for a conformation-specific mAb directed against the ligand-binding site (14) and a sequence-specific mAb directed toward the highly immunogenic region in the α-subunit of AcChoR (15). In the present study, we identified a 15-mer peptide that binds specifically to the pathogenic MIR-specific mAb 198. The binding affinity of this 15-mer parent peptide to mAb 198 was further improved by incorporating amino acid residues flanking this insert in the phage construct and additional cysteine residues at both ends of the resulting extended peptide, followed by oxidation to yield a cyclic disulfide-constrained peptide. The cyclic peptide was shown to very effectively inhibit the binding of mAb 198 to AcChoR and prevent accelerated degradation of AcChoR induced by mAb 198 in the AcChoR-expressing cell line TE671. Furthermore, this peptide was able to prevent the neuromuscular block and other symptoms of EAMG induced by this anti-MIR mAb.

Materials and Methods

Materials. The 15-mer phage-peptide library employed in this study (Chiron) was constructed by using phage M13 as described (11).

mAb 198 and other anti-MIR mAbs were kindly provided by S. Tzartos (Hellenic Pasteur Institute, Athens, Greece), prepared, and characterized as described elsewhere (10, 16, 17). mAb 198 is an IgG2a anti-MIR mAb derived from rats immunized with intact AcChoR isolated from human muscle. mAb 198 binds to AcChoR from human and rat muscle, and from *Torpedo* electric organs.

AcChoR was extracted from the electric organ of *Torpedo californica* and purified as described (18). A recombinant fragment of human AcChoR, comprising residues 1–205 of the extracellular domain of the α-subunit (hα1–205) has been cloned and expressed in our laboratory (19).

Peptides were synthesized by the Chemical Services Unit at the Weizmann Institute by using the standard solid-phase automated method. Cysteine-containing peptides were oxidized by stirring a 1 mM solution of peptide in PBS for 36 h at room

Abbreviations: AcChoR, acetylcholine receptor; MG, myasthenia gravis; EAMG, experimental autoimmune myasthenia gravis; MIR, main immunogenic reaction; ext. Pep., extended Pep.; cyc.ext.Pep., cyclic extended Pep; hMIR, human MIR.

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temperature. The extent of oxidation was monitored by measuring the free—SH content with Ellman's reagent.

Biopanning and Phage-ELISA. Purification and biotinylation of mAb 198 was performed as described elsewhere (14). Three rounds of affinity selection were performed as described by Devlin *et al.* (11), with minor modifications. A sample of phage library containing 1×10^{11} transducing units was incubated with biotinylated mAb 198 (concentrations ranging from 10 to 100 nM) overnight at 4°C in 40 μl of PBS containing 0.5% BSA. Petri dishes (60 mm, Nunc) were coated with streptavidin (10 μg/ml in 0.1 M $NaHCO_3$, 800 μl) overnight at 4°C and then blocked with PBS containing 3% BSA and 0.1 μg/ml streptavidin for 1 h at 37°C. The plates were washed six times with PBS containing 0.5% Tween-20. The mixture of phage with biotinylated mAb 198 was diluted with 500 μl of PBS containing 0.5% Tween-20 and added to the streptavidin-coated plates, followed by incubation for 30 min at room temperature with gentle shaking. Unbound phages were discarded and the plates were washed extensively five times with PBS and five times with PBS containing 0.5% Tween-20 at 2-min intervals. Bound phages from each of the plates were eluted with 600 μl of 0.1 M glycine·HCl (pH 2.2) for 10 min and immediately transferred to tubes containing 50 μl of Tris base. Eluted phages were amplified by infecting a log-phase culture of *Escherichia coli* K91*kan*. Phage particles were purified by standard polyethylene glycol/NaCl precipitation, quantified as transducing units (20), and used for subsequent rounds of panning. The antibody concentration was 100 nM in the first round and progressively reduced by one order in subsequent panning steps to select high-affinity clones. After the third panning, individual clones were grown in 96-well plates overnight at 37°C, and the phages were tested for their ability to interact with mAb 198 by phage-ELISA as described (14). Positive phage clones were sequenced and the deduced peptide sequences corresponding to the insert region were synthesized. The ability of the synthetic peptides or proteins to inhibit the binding of mAb 198 to positive phages was tested by ELISA (19).

Binding and Inhibition Experiments. Microtiter plates were coated with either *Torpedo* AcChoR (0.5 μg/ml), recombinant fragment Hα1–205 (5 μg/ml), or the cyclic peptide (2 μg/ml) in PBS overnight at 4°C. All subsequent steps were performed as described for inhibition of mAb 198 binding to phage-coated wells.

Competition Between Peptides and Rat Muscle AcChoR on mAb 198 Binding. Muscle extracts were prepared from leg muscles of denervated rats as described (21) and the AcChoR content was determined. An aliquot of rat muscle extract containing 0.5 nM AcChoR was incubated with 1 nM of ^{125}I-α-bungarotoxin for 4–6 h at 4°C. mAb 198 was diluted in PBS containing 5% normal rat serum (to yield a final mAb concentration of 1.5 nM) and preincubated for 1 h at 37°C with different concentrations of library-derived peptides in a total volume of 25 μl. To each tube, 100 μl of labeled muscle extract (100,000 cpm) was added. After overnight incubation at 4°C, 10 μl of goat anti-rat IgG was added and incubation continued for 4–6 h to precipitate the AcChoR–mAb 198 complexes. The samples were diluted with 1 ml of PBS containing 0.5% Triton X-100 and centrifuged. The pellets were washed twice with 1 ml of PBS containing 0.5% Triton X-100 and bound radioactivity measured. Nonspecific binding was determined in the absence of mAb 198. The extent of inhibition was assessed based on the decrease in the binding of mAb 198 in the presence of peptides relative to the binding in the absence of peptides. Antigenic modulation experiments were performed as described (19).

Table 1. Peptides selected from a 15-mer phage-epitope library

Peptide	Sequence*	Frequency†
Pep.1	PMTLPENYFSERPYH	40
Pep.2	KLRFPPHNLFTERML	3
Pep.3	HNIPNFLTPMNPYQT	4

*Amino acid sequences of peptide inserts expressed on the surface of the cloned phages were deduced from DNA sequencing of the insert.
†Frequency denotes the number of phage clones with identical inserts.

Prevention of EAMG. Passive transfer experiments were performed in 6-week-old female Lewis rats (weighing approximately 120 g) as reported (8) by injecting 90 μg of mAb 198 i.p. in PBS into rats. The effect of library-derived peptides was assessed by injecting a mixture of mAb 198 and competing peptides after preincubation for 1 h at room temperature. To test the ability of the cyclic peptide to combine with mAb *in vivo*, the cyclic peptide was injected either 1 h before or 15, 30, or 60 min after mAb 198 injection. The rats were monitored for myasthenic symptoms which were scored on the following scale: 0, normal; 1, weak grip or cry; 2, weakness, hunched posture at rest, decreased body weight, and tremor; 3, severe generalized weakness or paralysis; and 4, death. At the end of 26 h, the animals were killed and the AcChoR content in the leg muscles was determined as described (22).

Results

Isolation of Phages Displaying Specificity for mAb 198. In view of the pathogenic potential of anti-MIR antibodies in MG (23), we set forth to select MIR-mimetic ligands from a 15-mer random phage epitope library by using a MIR-specific mAb, i.e., mAb 198. After the third panning, the percentage of retained phage particles increased nearly 100-fold (0.0001 to 0.01%), which ensured the specific enrichment of mAb 198-binding phages. About 60% of the phages were positive for binding to mAb 198. DNA from 47 positive clones was sequenced. The deduced peptide sequences are shown in Table 1. Of these, 40 phage clones (Phage 1) exhibited the sequence PMTLPENYFSERPYH (Pep.1), 3 phages (Phage 2) represented a peptide sequence KLRFPPHNLFTERML (Pep.2), and the other four phage clones (Phage 3) represented a peptide sequence HNIPNFLTPMNPYQT (Pep.3). Alignment of the MIR region of AcChoR with the sequence exhibited by Phage 1 indicates that the sequence ^{69}PDDY^{72} of the human AcChoR α-subunit is homologous to the tetrapeptide sequence PENY, present in this peptide (residues 5–8, Table 1). The other two phage-derived sequences, Pep.2 and Pep.3, do not bear any significant homology to AcChoR. All three phages bound mAb 198 in a dose-dependent manner. The concentrations of mAb 198 that gave half-maximal binding to the corresponding immobilized phages were 5 nM for Phage 1 and about 50 nM for Phage 2 and Phage 3. Binding of all three phages to mAb 198 was highly specific, because they did not bind to a control-nonrelated mAb. Also, mAb 198 did not bind to phages selected by other nonrelated mAbs.

Inhibition of mAb198 Binding to Selected Phages by Synthetic Peptides. To ensure that the interaction between the selected phages and mAb 198 is caused by the insert sequences, peptides encompassing this region were synthesized. The ability of these peptides to compete with mAb 198 binding to Phage 1 was assessed. Whereas Pep.1 and Pep.2 inhibited the binding of mAb 198 to Phage 1 in a dose-dependent manner, Pep.3 demonstrated no inhibition (Fig. 1). The IC_{50} values for Pep.1 and Pep.2 were 100 μM and $\approx$300 μM, respectively. It should be pointed out that there is some notable homology between residues 5–12 of Pep.1

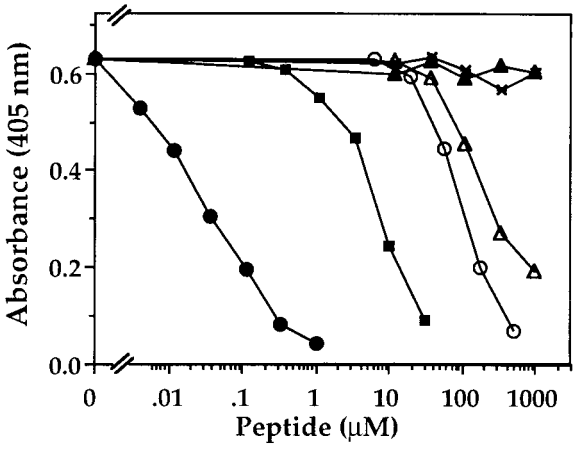

Fig. 1. Inhibition of mAb 198 binding to Phage 1, bearing the insert sequence PMTLPENYFSERPYH. Immobilized Phage 1 was incubated with 100 μg/ml of biotinylated mAb 198 alone or preincubated with Pep.1 (○); Pep.2 (△); Pep.3 (*); ext.Pep.1 (■); cyc.ext.Pep.1 (●); and the hMIR peptide, residues 67–76 (▲).

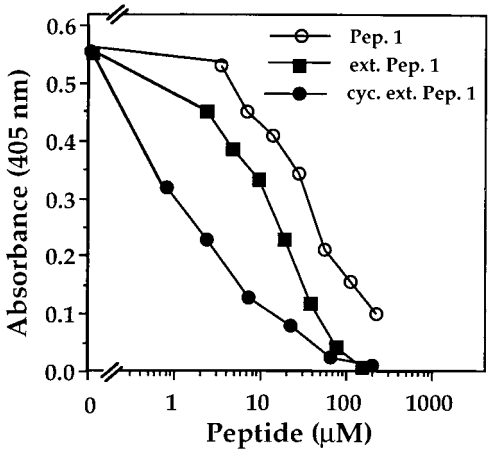

Fig. 2. Inhibition of mAb 198 binding to *Torpedo* AcChoR by library-derived peptides. Microtiter wells coated with *Torpedo* AcChoR (0.5 μg/ml) were treated with either biotinylated mAb 198 alone or after preincubation, with peptides. Bound mAb was detected by using streptavidin-alkaline phosphatase conjugate.

and 6–13 of Pep.2, with five identical residues (PxNxFxER; Table 1). Moreover, in the human MIR peptide, Pro-69 and Lys-76 (a positively charged amino acid) are spaced similarly to the Pro and Arg (also positively charged) in Pep.1 and Pep.2, respectively. This similarity may indicate the importance of these residues for the interaction of the peptides with mAb 198. The lower affinity of Pep.1 and Pep.2 for mAb198, when compared with the affinity of the parent phage (Phage 1) to this antibody, suggested that residues flanking this peptide in the phage may stabilize the conformation of the fused peptide, thus enhancing its affinity. In an attempt to improve the affinity of Pep.1 to mAb 198, we extended Pep.1 by including two residues (AE) at the N-terminus and four residues at the C-terminus (PPPP) that flank the inserted peptide in the phage library construct. As anticipated, the extended Pep.1 (ext.Pep.1; Table 2) inhibited the binding of mAb 198 to Phage 1 with an improved IC_{50} of 10 μM, which is 10 times better than the IC_{50} (100 μM) obtained with the parent 15-mer peptide (Fig. 1). Despite the improved affinity, the IC_{50} value is yet three orders of magnitude higher than the concentration of mAb 198 required for half-maximal binding to Phage 1. This may imply that conformational constraints imposed by the phage are still not met by the ext.Pep.1. Considering the facts that mAb 198 is highly conformation dependent and conformational epitopes may be better mimicked by constrained peptides, we have constrained the ext.Pep.1. by addition of cysteine residues at both ends and subsequent oxidation. This cyclic peptide (cyc.ext.Pep.1) exhibited a much better affinity to mAb 198 (IC_{50} 40 nM, Fig. 1) that is similar to the half-maximal binding of mAb 198 to Phage 1 (50 nM).

Inhibition of mAb 198 Binding to AcChoR by Library-Derived Peptides. To determine whether the library-derived synthetic peptides mimic MIR in its native context, the ability of the peptides to inhibit the binding of mAb to *Torpedo* AcChoR was assessed by ELISA. All three library-derived peptides inhibited the interaction of mAb 198 with *Torpedo* AcChoR in a concentration-dependent manner (Fig. 2). The best inhibition was obtained with the 23-mer cyc.ext.Pep.1, CAEPMTLPENYFSERPYHPPPC (IC_{50} 2 μM). Because mAb 198 was raised against human AcChoR, we wanted to test the peptide inhibition of the binding of this mAb to human AcChoR. Although human AcChoR is not available in a purified form, we have studied the potential of the library-derived peptides to inhibit the binding of mAb 198 to a human recombinant fragment of AcChoR. This fragment, designated Hα1–205, encompasses the entire extracellular domain of the α-subunit of AcChoR and has been cloned in our laboratory (19). All three peptides, namely, the library peptide Pep.1, ext.Pep.1, and cyc.ext.Pep.1 (in particular the last), demonstrated a better inhibition of the binding to Hα1–205 than that of the binding to *Torpedo* AcChoR (Fig. 3). The cyc.ext.Pep.1 blocked the binding of mAb 198 to Hα1–205 with an IC_{50} of 30 nM. Pep.2 and Pep.3 exhibited weak inhibitory ability; nevertheless, they both were able to inhibit the binding of mAb 198 to

Table 2. Sequences of peptides derived from Pep.1

Peptide	Sequence
Pep.1	PMTLPENYFSERPYH
ext.Pep.1	AE. . . . . .PPPP
ext.Pep.1 F11H	AE. . . . . H. . . . . PPPP
cyc.ext.Pep.1	CAE. . . . . .PPPPC
hMIR*	WN.DD.GGIK

*hMIR refers to the main immunogenic region of the human AcChoR α-subunit, encompassing residues 67–76.

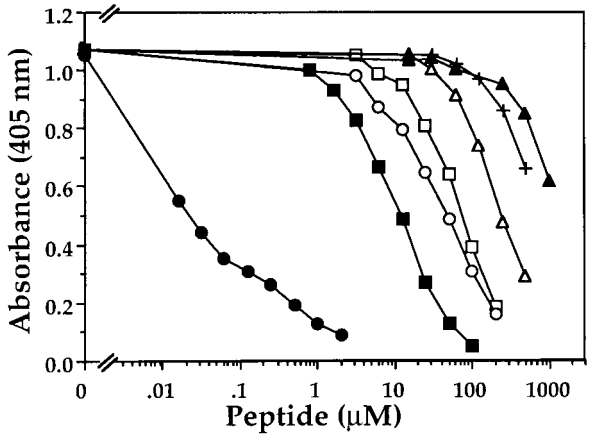

Fig. 3. Inhibition of mAb 198 binding to recombinant human AcChoR fragment (hα1–205) by synthetic peptides. Microtiter wells coated with 0.2 μg/ml of hα1–205 were treated with biotinylated mAb 198 either alone or after preincubation with Pep.1 (○); Pep.2 (△); Pep.3 (+); ext.Pep.1 (■); cyc.ext.Pep.1 (●); hMIR peptide (▲); and ext.Pep.1F11H (□).

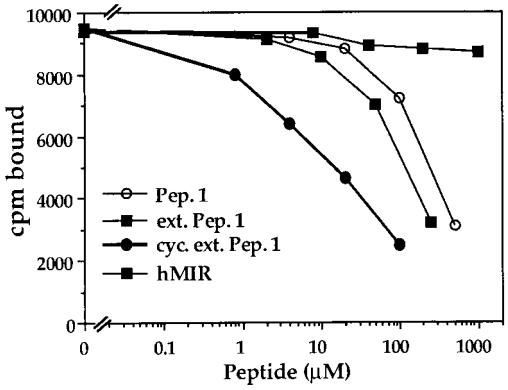

Fig. 4. Inhibition of mAb 198 binding to rat AcChoR by synthetic peptides. Rat AcChoR in muscle extracts was labelled with ^{125}I-α-bungarotoxin and incubated overnight at 4°C with mAb 198 either alone or after preincubation with synthetic peptides. Receptor-bound mAb was quantified after precipitation with anti-rat IgG.

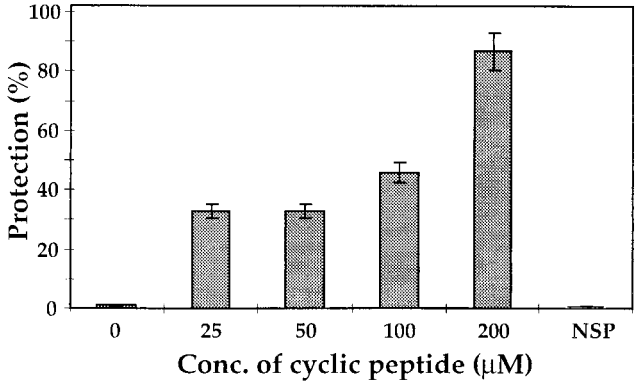

Fig. 5. The cyclic peptide inhibits AcChoR degradation induced by mAb 198. TE671 cells were treated with 1 μg/ml of mAb 198 alone or after preincubation with indicated concentrations of cyc.ext.Pep.1. After a 3-h incubation at 37°C, cell surface AcChoR content was quantified by ^{125}I-α-bungarotoxin binding.

hα1–205 to a higher extent than a peptide corresponding to the sequence of the human MIR region (designated hMIR; residues 67–76 of the α-subunit).

Substitution of Gly-73 in the hMIR peptide for His was reported to result in a threefold increase in the binding of hMIR to mAb 198 (24). To test whether such a substitution would be beneficial also for the library-derived peptides, we have introduced a substitution in an analogous position (Phe to His; Table 1) in ext.Pep.1 (ext.Pep.1F11H). As shown in Fig. 3, such a substitution resulted in a decrease in the inhibition of the binding of mAb 198 to hα1–205.

To further examine the specificity of the library-derived peptides, we have tested their capacity to inhibit the binding of mAb 198 to native mammalian receptor. For that, we have assessed the ability of the peptides to inhibit the binding of mAb 198 to rat muscle AcChoR, which is highly homologous to human AcChoR. As depicted in Fig. 4, the cyc.ext.Pep.1 was most efficient in this inhibition (IC_{50} 10 μM). This inhibition is about 25-fold lower than that obtained (IC_{50} 0.4 μM) with the human AcChoR fragment.

The Cyclic Library-Derived Peptide Prevents Antigenic Modulation of AcChoR in Human TE671 Cells Induced by mAb 198. Accelerated degradation of the receptor induced by anti-MIR autoantibodies leads to muscle AcChoR loss in myasthenic animals and patients (6, 7). To assess the potential of the ext.cyc.Pep.1. as a candidate for *in vivo* protection of EAMG, we examined its ability to protect cell surface AcChoR against the accelerated degradation induced by mAb 198. When TE671 cells were incubated with 1 μg/ml of mAb 198, almost 60% of the receptors are degraded in 3 h; however, preincubation of mAb 198 with increasing concentrations of the cyc.ext.Pep.1 protects AcChoR from the accelerated degradation in a dose-dependent manner (Fig. 5). At a concentration of 200 μM, more than 95% protection could be achieved. None of the other tested peptides showed any significant protective activity. It should be noted that complete oxidation of the peptide was essential to confer efficient protection against antigenic modulation induced by mAb198.

Interaction of the Cyc.Ext.Pep.1 with Other Anti-MIR mAbs and Polyclonal Anti-AcChoR Sera of Myasthenic Rats. To evaluate the possible applicability of the cyc.ext.Pep.1, we tested its potential to interact with additional anti-MIR mAbs and polyclonal antibodies of rats with EAMG. As depicted in Fig. 6*a*, mAbs 28, 42, 22, 35, and 50, all of which were demonstrated to react also with the hMIR peptide (residues 67–76 of the α-subunit) (10, 24, 25) bind, albeit to a lower extent than mAb 198, to the cyc.ext.Pep.1. As was expected, a monoclonal anti-AcChoR (mAb 155, Fig. 6*a*) directed to an epitope in the cytoplasmic region of the receptor α-subunit (10), as well as two additional mAbs (mAb 5.5 and mAb 5.14; data not shown) that are not MIR-specific (14, 15), did not bind to the cyc.ext.Pep.1. mAb 28 and 42, which gave a higher reactivity than the other mAbs, comes from rats immunized with eel AcChoR, whereas mAb 198 comes from rats immunized with human receptor. Both were shown to bind to mammalian AcChoR.

In addition, the cyc.ext.Pep.1 exhibited significant binding to total IgG as well as to the disease-specific isotype IgG2a, in sera obtained from rats with EAMG (clinical score 2–3) 8 weeks after immunization with *Torpedo* AcChoR (Fig. 6*b*).

The Cyclic Library-Derived MIR-Mimotope Prevents EAMG Passively Transferred by mAb 198. Once cyc.ext.Pep.1 was shown to interact specifically and with high affinity with mAb 198 in *in vitro* experiments, we tested whether this peptide might block the induction of EAMG by mAb 198 to verify its possible therapeutic potential. Rats were administered i.p. with mAb 198 (90 μg per rat) either alone or in a mixture with the library-derived peptides (1 mg of Pep.1, ext.Pep.1, or cyc.ext.Pep.1). In rats injected with mAb 198 alone, severe myasthenic symptoms were observed 16–26 h following antibody transfer. As seen in Fig. 7 (*Upper*), this neuromuscular block was abolished by the cyc.ext.Pep.1. A partial protection was observed by the linear ext.Pep.1, and the original library peptide (Pep.1) did not at all prevent the induction of EAMG by mAb 198. Administration of the cyc.ext.Pep.1 alone did not induce any myasthenic symptoms. It should be noted that the cyc.ext.Pep.1 conferred complete protection against EAMG even when injected 1 h before or immediately after the injection of mAb 198. Administration of the peptide 15 or 30 min after mAb 198 injection reduced the symptoms to clinical score 2, whereas the control animals were all dead (clinical score 4). The protective effect offered by the cyc.ext.Pep.1 is further confirmed by the inverse correlation between the mean clinical score and the AcChoR content in the control (injected with mAb alone) and protected (injected with mAb and cyc.ext.Pep.1) animals. As reported, control rats injected with mAb 198 alone lost almost 60% of their muscle AcChoR content. On the other hand, rats administered with mAb 198 together with the cyc.ext.Pep.1 maintained their receptor content close to that of noninjected rats. The linear ext.Pep.1, which conferred partial protection, resulted in a loss of 35% of the receptor content. These results once again emphasize the importance of

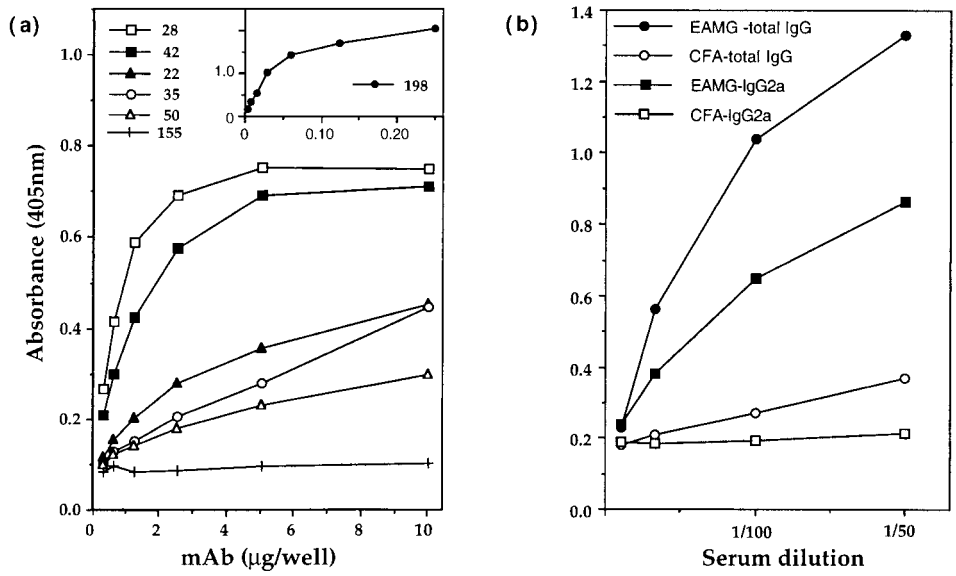

Fig. 6. Binding of the cyc.ext.Pep.1 to anti-MIR mAbs (*a*) and rat anti-AcChoR sera (*b*). Peptide-coated plates were incubated overnight with dilutions of indicated mAbs (*a*) and sera from EAMG rats, or control complete Freund's adjuvant-immunized rats (*b*). Bound antibody was detected with anti-rat IgG conjugated to alkaline phosphatase or with IgG2a-specific biotinylated antibodies.

constrained conformation for efficient interaction with mAb 198.

Discussion

Current immunotherapeutic protocols for myasthenia gravis employ global immunosuppressive agents which are nonspecific and, hence, might be accompanied by side effects and toxicity. An ideal treatment would influence only the disease-associated autoantibodies. Theoretically, this can be achieved by employing surrogate antigens which could inhibit the binding of the pathogenic antibodies to muscle AcChoR and thus prevent the impairment of neuromuscular transmission. With this view in mind, we have previously attempted to modulate the anti-AcChoR response by employing denatured derivatives of *Torpedo* AcChoR or recombinant fragments of the α-subunit of human AcChoR (19, 26). Although these derivatives are effective in modulating EAMG, the use of small synthetic ligands capable of immunoneutralizing pathogenic mAbs could be advantageous because they are usually less immunogenic and it is unlikely that they themselves will induce the disease. Furthermore, because it is well established that a major portion of autoantibodies in MG are against the MIR region, use of MIR-mimetic ligands might eliminate some of the undesirable autoantibodies. Because of the conformational stringency of the epitopes for anti-MIR antibodies, linear synthetic peptides corresponding to the MIR region of AcChoR were not successful in neutralizing them. We thus have used the phage-peptide library to select candidate peptides which could mimic the MIR region. The high conformational dependency of mAb 198 was evident from our unsuccessful attempts to select specific mimotopes for this mAb from several linear libraries as well as from a 7-mer disulfide-constrained library.

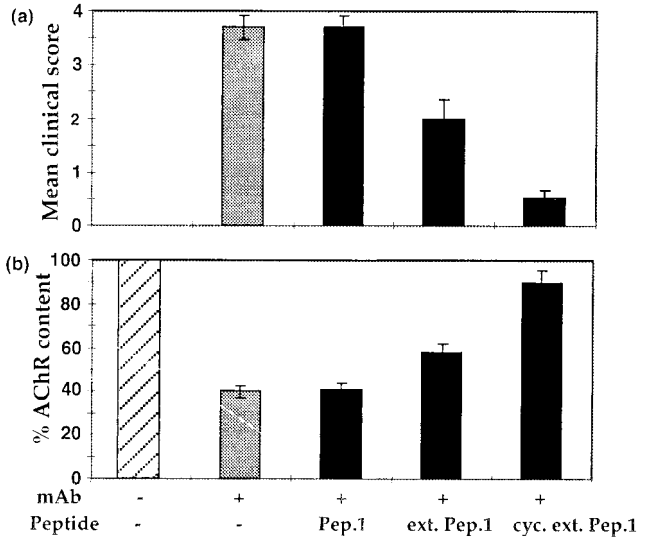

Fig. 7. Prevention of EAMG-induced by mAb 198 with library-derived synthetic peptides. Lewis rats were injected i.p. with 90 μg of mAb 198 either alone or after preincubation with 1 mg of peptide. Clinical symptoms and muscle AcChoR content were determined as described in *Materials and Methods.* Values (means ± SEM) represent results obtained from three different animals in two independent sets of experiments.

By screening a 15-mer library, we obtained three different peptides capable of specifically binding to mAb 198 with a rather low-binding affinity. We chose the best of the three peptides with the sequence PMTLPENYFSERYPH (Pep.1) as a lead peptide to improve its affinity to mAb 198. Comparison of this peptide with the hMIR sequence, WNPDDYGGIK (residues 67–76 of the human AcChoR α-subunit), suggests that the sequence PENY within Pep.1 is homologous to the sequence ^{69}PDDY^{72} of the hMIR sequence, which was shown to be critical for antibody binding (27). Studies employing synthetic peptides and site-directed mutants of the AcChoR α-subunit demonstrate that residues Asn-68, Asp-70, Asp-71, and Tyr-72 play an important role in defining the binding specificity of MIR to mAb 198 (27). It should be noted, however, that residues corresponding to Asn-68 and Asp-71 in the human AcChoR α-subunit are replaced in the library peptide by Leu and Asn, respectively. Nevertheless, the library peptide, Pep.1, as well as Pep.2, were better inhibitors of the binding of mAb 198 than the receptor-derived MIR peptide encompassing residues 67–76 (Figs. 1–4).

Thus, the library peptides, despite having a different sequence, mimic the conformation of the MIR region in the native context.

Constrained peptide libraries were demonstrated to be more suitable for selection of ligands for conformation-dependent antibodies (28). However, we failed to select a ligand for mAb 198 by using a 7-mer constrained library. As an alternative, we have constrained the ext.Pep.1 by incorporating flanking cysteines on both ends and subsequent oxidation. Indeed, the resulting cyc.ext.Pep.1 was a much better inhibitor, and inhibited the binding of mAb 198 to the recombinant receptor fragment with an IC_{50} of 40 nM. Although optimal conformation of cyclic peptides is dependent on the ring size, one may need to try several constrained libraries to successfully identify mimotopes for conformation-dependent mAbs. Therefore, as an alternative to the application of constrained-peptide libraries in cases where they did not prove successful, we suggest a scheme of (*i*) selection of peptides with moderate affinity from linear peptide libraries, (*ii*) elongation of the parent peptide with flanking residues of the phage construct, and (*iii*) cyclization. We have indications that this approach has been successful in some additional cases and therefore believe that it may provide a general means for improving the affinity of peptides selected from linear phage epitope libraries.

We demonstrated that the cyc.ext.Pep.1 efficiently inhibits mAb 198 binding to different receptor preparations such as *Torpedo* AcChoR, the human AcChoR fragment, and rat AcChoR. In addition, the cyc.ext.Pep.1 prevents AcChoR loss induced by mAb 198 in TE671 cells and prevents the induction of EAMG by passive transfer of mAb 198. It should be noted that the reduced noncyclic form of the peptide was a markedly weaker inhibitor than the cyc.ext.Pep.1. This indicates that constrained conformation is critical for specific recognition by mAb 198. It is likely that cyc.ext.Pep.1 is more stable and therefore more suitable for *in vivo* application. In our case, we constrained the linear peptide by a disulfide bond; nevertheless, it is possible that other strategies of cyclization might be adopted to yield more stable derivatives with improved *in vivo* stability.

In spite of the heterogeneity of the MIR-specific antibodies (2, 29), some crossreactivity of the cyc.ext.Pep.1 selected by mAb 198, has been observed with several other anti-MIR mAbs as well as with sera from rats with EAMG. Such a crossreactivity might make this peptide, or modified forms of it, suitable for the treatment of EAMG induced by immunization with AcChoR. A systematic substitution in the present cyc.ext.Pep.1 may result in variants with specificity for other anti-MIR antibodies. It is also worthwhile to attempt selecting mimotopes for additional anti-MIR mAbs and search for a common motif. In addition, one might also search for mimotopes that recognize public epitopes that are shared by various anti-AcChoR antibodies in MG patients. If a combination of such mimotopes blocks the binding of a major fraction of autoantibodies in the serum of EAMG rats or MG patients, then such peptides can possibly have a potential application for antigen-specific treatment for MG.

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