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Antibodies to acetylcholine receptor in parous women with myasthenia: evidence for immunization by fetal antigen

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Running title: Fetal-specific antibodies in arthrogryposis

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Abstract

The weakness in myasthenia gravis (MG) is mediated by autoantibodies against adult muscle acetylcholine receptors (AChR) at the neuromuscular junction; most of these antibodies also bind to fetal AChR which is present in the thymus. In rare cases, babies of MG mothers, or even of asymptomatic mothers, develop a severe developmental condition, arthrogryposis multiplex congenita (AMC), due to antibodies that inhibit the ion channel function of the fetal AChR, whilst not affecting the adult AChR. Here we show that these fetal AChR inhibitory antibodies are significantly more common in females sampled after pregnancy than in those who present before pregnancy, suggesting that they may be induced by the fetus. Moreover, we were able to clone high-affinity combinatorial Fab antibodies from thymic cells of two MG mothers with AMC babies. These Fabs were highly specific for fetal AChR and did not bind the main immunogenic region (MIR) that is common to fetal and adult AChR. The Fabs show strong biases to VH3 heavy chains, and to a single Vκ1 light chain in one mother. Nevertheless, they each show extensive intraclonal diversification from a highly mutated consensus sequence, consistent with antigen-driven selection in successive steps. Collectively, our results suggest that, in some cases of MG, initial immunization against fetal AChR is followed by diversification and expansion of B cells in the thymus; maternal autoimmunity will result if the immune response spreads to the main immunogenic region and other epitopes common to fetal and adult AChR.

Abbreviations:- α-BuTx, α-Bungarotoxin: AChR, acetylcholine receptor: AMC, arthrogryposis multiplex congenita: CDR, complementarity-determining region: EOMG, early-onset myasthenia gravis: FWR, framework: GC, germinal center(s): MIR, main immunogenic region: R:S, replacement: silent.

Keywords: acetylcholine receptor, fetal antigen, arthrogryposis multiplex congenita, myasthenia gravis, autoimmunity

Introduction

Autoimmune disorders collectively cause much suffering and disability and many are particularly common in women of child-bearing age. The muscle weakness in the majority of myasthenia gravis (MG) patients results from autoantibodies to the acetylcholine receptor (AChR) which cause AChR loss, mainly by complement-mediated damage to the post-synaptic membrane and/or accelerated AChR degradation (reviewed by Drachman,1994), Willcox and Vincent,1988). The AChR consists of two α subunits and one β, γ and δ in the fetus (Unwin, 2000). Together, these subunits form a cation-specific ion channel that opens when ACh binds to the two sites at the α/δ and either the α/γ or α/ε interfaces (Unwin, 2000). From about 30 weeks' gestation in humans, the 'adult'-specific ε subunit gradually replaces the γ subunit (Hesselmans et al, 1993), but the fetal isoform continues to be expressed on rare myoid cells in the adult thymic medulla (Schluep et al, 1987).

Early-onset MG (before age 40; EOMG) shows a ~3:1 female bias and a strong HLA-DR3-B8 association. The thymus is apparently an important site of autoimmunization (Drachman, 1994; Willcox and Vincent 1988; Kornstein et al, 1984; Bofill et al, 1985; Schluep et al, 1987; Sims et al, 2001); thymectomy is often beneficial and, in most EOMG cases, the thymus is invaded by lymph node-like T cell areas and germinal centers (GC; (Willcox and Vincent, 1988; Bofill et al 1985; Kornstein et al 1984), many of which show AChR-specificity (Sims et al 2001). GC are well known sites of B cell memory generation and of antibody diversification by antigen-selection of somatic variants (Sims et al 2001; MacLennan 1994). Moreover, there is selective activation of thymic plasma cell responses, with spontaneous production of anti-AChR antibodies (Willcox and Vincent 1988), which show specificities similar to those in the patients' blood (Heidenreich et al 1988). The anti-AChR antibodies in typical MG patients are very heterogeneous (Vincent et al 1987) and antibodies cloned from the thymus of typical EOMG patients show a range of specificities resembling those in the donors' sera (Graus et al 1997; Farrar et al 1997; Serrano et al 1994).

About 8% of MG mothers have babies with transient neonatal MG, and their sera tend to have higher antibody titers against fetal than adult AChR (Vernet der Garabedian et al 1994), consistent with a role for thymic AChR in inducing their disease. In some rare examples, babies of MG mothers are born with severe developmental abnormalities, usually described as *arthrogryposis multiplex congenita* (AMC; reviewed in Hall and Vincent, 2001), which includes multiple joint contractures, hypoplasia of the lungs, other malformations and often fetal or neonatal death. The serum of these MG mothers, and others who are asymptomatic (Vincent et al 1995), contains antibodies that strongly and very selectively inhibit the ACh-triggered ion channel function of fetal AChR (Riemersma et al 1997). These inhibitory antibodies persist in the maternal circulation and can transfer a similar condition to the pups after injection into pregnant mice (Jacobson et al 1999a). Interestingly, the first child of mothers of AMC babies is often unaffected and, when present, MG may not be clinically evident in the mother at the time of the first affected birth (see Polizzi, Huson and Vincent, 2000), suggesting that the maternal immune system may first be sensitized to AChR from the fetus. Moreover, the dominance of fetal AChR-specific antibodies in these women (Riemersma et al 1997) suggested that the B cell response might be clonally restricted.

Here we examined the relationship between inhibitory antibodies and parity in MG patients, and then took advantage of the fact that EOMG patients are treated by thymectomy, providing us with thymic B cells and plasma cells from which to clone and recombine fetal-AChR specific antibodies. We selected combinatorial Fabs from two MG mothers with high levels of fetal AChR-specific antibodies, whose babies had severe AMC. These Fabs proved to be strongly biased towards fetal AChR and were each dominated by one clone with extensive somatic diversification from an already highly mutated consensus sequence. These results suggest that the fetus could be responsible for immunizing the mother during pregnancy, with further diversification occurring subsequently in the thymus.

Results

Antibodies inhibiting fetal AChR function in MG. We searched our records for women with MG who had had children before their first available serum sample. We found twelve women with generalized MG who had had 1-3 children each by the time of sampling, which was before thymectomy or immunosuppressive treatment (Parous MG; see Table 1); in all but one case, MG presented during or after pregnancy. We compared the results with those of 12 women who had not had children at the time of sampling (Non-parous MG), 11 male MG patients and 12 healthy controls. Two AMC-M sera were used as positive controls. As expected, the age at MG onset was higher in the Parous MG than the Non-parous MG women (mean ± SD; 30.7 ± 7.8 compared with 19.9 ± 5.3), but there was no substantial difference in the total levels of anti-AChR antibodies on routine testing (22.26 ± 10.6 nM compared with 26.05 ± 12.7 nM). Male MG patients were of a similar age to parous females (30.9 ± 8.4) and with similar anti-AChR values (18.5 ± 12.7). To assay the levels of fetal-AChR inhibitory antibodies, we measured the effects of the sera on agonist-induced ^{22}Na flux into TE-671 cells. Healthy sera did not inhibit flux appreciably (0.02 ± 0.58% compared to results in Hepes-Locke buffer alone). Inhibition of flux by the Parous MG sera was greater than that by the Non-parous (p=0.023; Mann Whitney one-tailed; Fig 1) or male MG sera. These results suggest that pregnancy can influence the specificity of AChR antibodies in MG patients, and may initiate the response in some susceptible individuals.

Cloning anti-AChR Fabs from thymic combinatorial libraries. In order to examine the clonal origins of fetal-specific AChR antibodies, we characterized Fabs cloned from unamplified VH/Vκ cDNA libraries prepared from thymic cells of AMC-M2 and AMC-M6, after screening 2.0-2.5 x 10^5 clones. It was relatively easy to detect AChR-specific plaques by blotting the expressed Fabs with ^{125}I-αBuTx-AChR solubilized from human muscle, and to clone the positives by further rounds of screening (Fig 2a); the results are summarized in Table 2. Fetal AChR inhibitory antibodies competed with one of the two α-BuTx-binding sites (Riemersma et al 1997), leaving the second site available for detection with ^{125}I-α-BuTx.

Therefore, in order to clone Fabs that might compete with α-BuTx for binding to the fetal AChR, we re-screened the AMC-M2 library with unlabeled muscle extract, allowing the AChR to bind before we applied the ^{125}I-α-BuTx. A further 25 clones were thus isolated and two characterized in detail. We also prepared a parallel VH/Vλ library from AMC-M2 and isolated another 25 clones (Table 2).

Specificity of AMC-M Fabs for fetal AChR. Both AMC-M2 and AMC-M6 libraries yielded Fabs that efficiently immunoprecipitated ^{125}I-α-BuTx-human AChR, in most cases precipitating all of the available receptor. Examples of titrations of the Fabs are shown in Fig 2b and c; the efficient binding by monomeric Fabs of soluble AChR (at ~500 pM final concentration) indicates a high affinity. The muscle extracts used for screening and precipitations were from amputees with ischemic disease; although these have a preponderance of fetal over adult AChR (Vincent and Newsom-Davis 1984), antibodies to the α, β or δ subunits bind similarly to both isoforms (Tzartos et al 1998; Fostieri, Beeson and Tzartos 2000). We therefore tested the Fabs against AChR extracted from TE671 cells that express only fetal AChR, or from a TE671 subline that expresses predominantly (>90%) adult AChR (Beeson et al 1996). The two AMC-M sera preferred the fetal isoform, although they also showed some reactivity with adult AChR (Figure 3a). However, all of the Fabs, with one exception (Fab 8H/K from AMC-M6), bound almost exclusively to fetal AChR. Even the low apparent reactivity with the adult AChR preparation could be due to the remaiing 10% of fetal AChR (Beeson et al 1996).

To confirm this striking finding, we also tested the Fabs' ability to block the binding of mAbs specific for human fetal AChR γ subunits. Both AMC-M2 and AMC-M6 serum antibodies blocked binding of each of the two mAbs specific for fetal AChR (Figs 3b and c), as did all the Fabs except 8K from AMC-M6, which instead blocked binding of the mAb with β subunit-specificity (Fig 3c). These experiments confirm the high affinity for fetal AChR of the Fabs and most of the serum antibodies, and their probable γ subunit-specificity.

It was also important to test the Fabs for inhibition of fetal AChR ion channel function. Disappointingly, only Fab 35K from AMC-M2 showed appreciable activity and that only at relatively high Fab concentrations (50% inhibition at 100 μl of Fab). There was no effect on adult AChR (data not shown). In parallel experiments, cross-linking the cloned Fabs with secondary antibodies did not increase the degree of inhibition. Indeed, inhibition of function did not require divalent antibodies, since it was readily measurable with monovalent Fabs prepared from both donors' serum Ig (data not shown).

Sequences of the AMC-M2 Fab VH and Vκ genes. The V-genes were sequenced and compared with those in the human Ig VBASE directory (Tomlinson et al 1997) to identify both the closest germline sequence and the number of somatic mutations (Table 3). All the AMC-M2 Fabs were specific for fetal AChR and used the same VH3-07/D?/JH6b combination, regardless both of the screening procedure used to identify them (Table 2) and of their exact κ (Table 3) or λ light chain partner (not shown). They had the same CDR3 length and the majority shared 32 'consensus' VH mutations, demonstrating a common clonal origin from a previously-mutated progenitor. Replacement:silent (R:S) ratios were higher in the first two hypervariable (CDR) loops than in the framework regions (FWR), strongly suggesting antigen-driven selection for antibody specificity/affinity.

By contrast, the AMC-M2 kappa light sequences were heterogeneous; despite their similar binding specificity, these Fabs used a variety of Vκ (Table 3), which varied substantially both in numbers of mutations and R:S ratios. Although two Fabs used the Vκ 02/12 gene, their different Jκ usage demonstrates that they are independent gene rearrangements. This contrasting VH restriction and Vκ diversity suggests that the heavy chain was primarily responsible for the fetal AChR-binding specificity.

Recurring VH and Vκ usage by AMC-M6 Fabs. Table 3 also summarizes the genes encoding the second donor's anti-AChR Fabs. Fab 8H/K is the first human AChR β subunit-specific autoantibody to be cloned. It uses the relatively uncommon VH4-61 gene in

combination with the commonly expressed VK1 02/12 Vκ gene. Both the heavy and light chains are highly mutated, with higher R:S ratios in the CDR than the FWR regions (Table 3). One of the fetal AChR-specific Fabs (Fab 1H/K) used a highly mutated VH3-23/DH3.3/JH6b plus a Vκ4 B3/Jκ 3 gene (Table 3). All of the others used the same pair of VH3-21/D?/JH5b heavy and Vκ 02/12 light chain genes, again with Jκ4 (Table 3); the frequency of base differences between these sequences is significantly above the PCR error rate for the Taq polymerase used (Chazenbalk et al 1993), but the VHs and Vκs are clearly clonally-related.

The extent of somatic mutation shows that each is clearly derived from a highly mutated progenitor (with 44 consensus substitutions in the VH and 25 in the Vκ). The R:S ratios for the heavy chain gene are moderate, because the number of VH mutations approaches saturation (often 2 or even 3 per codon). Their closest germline counterparts and their clonal relationships are shown in Fig 4; the pattern is essentially similar for the VH sequences of the AMC-M2 Fabs (not shown). In the genealogical trees for both heavy and light chains, the branching patterns, with variable numbers of successive mutations separating each sequence, are clear evidence of further antigen-driven clonal proliferation and somatic mutation; moreover each stems from an already-mutated progenitor. Notably also, since neither this heavy nor this light chain was found with other partners, they could both be derived from the same progenitor B cell.

Convergent mutations suggesting common fetal AChR-specific selection processes. We saw recurring replacements in the three independent fetal AChR-specific VH3 genes and especially in the Vκ 02/12 sequences. In brief, there was a CDR1 ^{31}S→T substitution in all VH3 Fabs from AMC-M2 and AMC–M6. Even more strikingly, among the Vκ 02/12 light chains, AMC-M2 13K and the majority of the AMC-M6 Fabs not only use Jκ4 but also have common ^{22}T→S, ^{27}Q→E, ^{28}S→T and ^{53}S→T replacements. The former three contribute to the CDR1 to form a ^{22}SRASET^{28} motif that is found in only two other human κ sequences in GenBank. Moreover, these recurring H and L chain mutations were not seen in another fetal

AChR-specific Fab from a non-parous EOMG female (Farrar et al 1997) or in the very different AChR β-specific Fab-8 from AMC-M6 (data not shown).

Discussion

We have shown, for the first time, that antibodies inhibiting the ion channel function of fetal AChR are common not only in mothers of AMC babies (Riemersma et al 1997), but also in women who developed MG after pregnancy. By contrast, such antibodies were uncommon in women who presented before pregnancy. In addition, the many high affinity Fabs that we cloned from the thymus of two AMC mothers showed a very strong preference for fetal AChR and a biased usage of VH3 genes. The striking diversification of the Fabs in each woman from a common, but already highly mutated, germ-line sequence, shows that the autoantibodies can arise via successive rounds of antigen-driven selection in a few clones. These results suggest a scenario in which oligoclonal responses to fetal AChR, including some that block fetal AChR function, are the initiating event in women who develop MG after pregnancy, irrespective of whether the level of these antibodies is sufficient to cause AMC. Immunization by the fetus is, therefore, another possible contributory factor to the female bias evident in many autoimmune diseases, in addition to the established hormonal influences (Whittacre 2001) and the possible rôles of fetal↔maternal microchimerism (Hall et al 1995; Maloney et al 1999; Nelson 2001).

The Fabs that we cloned from the two unrelated AMC-mothers show remarkable similarities. They clearly have high affinities, since they immunoprecipitate AChR at ~500 pM and, despite being monomeric, they also efficiently block binding of bivalent mAbs. In theory, the predominance of fetal AChR in the ischemic muscle extracts we used (Vincent and Newsom-Davis 1984) might have created a bias in screening towards fetal AChR-binding Fabs. However, the MIR on the α subunits is thought to be the target of the majority of AChR antibodies in MG (Tzartos et al 1998); although some may have a preference for the fetal isoform (Fostieri, Beeson and Tzartos 2000), we should also have been able to detect them with adult AChR, and to inhibit them with the anti-MIR mAb. In fact, the almost

exclusive recognition of fetal, rather than adult, AChR contrasts sharply with Fabs cloned from the thymus of a more typical non-parous young female MG patient under the same conditions. In Farrar et al (1997) we obtained four distinct Fabs; three of them bound adult and fetal isoforms impartially and competed with mAbs specific for the main immunogenic region (MIR) on the alpha subunits. Only the fourth, which used another VH3 gene, DP58, showed similar fetal-AChR specificity to the present Fabs. In another study which also used predominantly fetal AChR for selection (Graus et al 1997), only anti-MIR Fabs were obtained. Thus we do not feel that the selection with ischaemic muscle AChR explains the bias towards fetal AChR. Although we were not able to generate a library from a control thymus, in a study of Fabs derived from an MG thymoma library, we found only one with AChR reactivity but a large number binding to interferon alpha, apparently reflecting the spontaneous production of anti-interferon alpha antibodies but not AChR antibodies by the patient's thymic lymphocytes (H Shiono, Y Wong, Willcox and Vincent, in preparation). Thus the selection process is antigen-specific and depends on the presence of the relevant heavy and light chain genes in the thymic tissue.

We had hoped to clone Fabs that inhibited fetal AChR ion channel function, since these antibodies are clearly present in the AMC-M sera (Riemersma et al 1997) and in 5/12 of the parous-MG women, and we found that monomeric serum Fabs retain inhibitory activity. Although the main ACh-binding sites are on the α subunits, they lie at the α/δ and α/γ interfaces: the distinctive α/γ site of the fetal isoform appears to be the target for the inhibitory antibodies (Riemersma et al 1997). Similar antibodies were found in one MG serum using rat fetal AChR in an early study (Weinberg and Hall 1979), although they bound to both adult and fetal isoforms when human AChR was used (Burges et al 1990). These inhibitory antibodies compete with ^{125}I-α-BuTx for binding to one of its two sites, making their detection with ^{125}I-α-BuTx-AChR more difficult, although we tried to overcome this difficulty by overlaying the filters with unlabeled AChR and adding ^{125}I-α-BuTx subsequently. However, despite their potency in causing AMC, they constitute only about 5% of the total anti-AChR antibodies in either these patients' thymic culture supernatants (not shown), or

their sera (A Vincent, unpublished calculations from data in Riemersma et al 1997). This probably explains their rarity among our cloned Fabs.

The efficient screening and cloning of Fabs binding specifically to fetal AChR implies a striking dominance of this specificity in the AMC mothers' thymic cells. Furthermore, we found a single dominant family of clonally related Vκ sequences in AMC-M6 and of VH3 sequences in both patients. In AMC-M2, the same clonally related VH3-07 sequences predominated whether they were isolated together with κ or λ light partners and irrespective of the screening procedure used (Table 2). A parallel restriction in Fabs recognizing a minority epitope, contrasting with heterogeneity of those against a dominant region, has recently been reported for thyroid peroxidase in a patient with Graves' disease (Pichuri et al 2001). The VH3 germline genes of our two clonal families are both commonly used by normal blood B cells (de Wildt et al 1999), as is the Vκ1 02/12 that predominated in AMC-M6 and recurred in AMC-M2 (De Wildt et al 1999; Foster et al 1997). A PCR bias seems very unlikely since the five non-specific Fabs isolated from our AMC-M2 VH/Vκ library did not include the VH3-07, VH3-21 or Vκ1 02/12 genes and VH3 genes are not over-represented in Fabs cloned, using the same primers, from other MG patients against other antigens (Y Wong, N Willcox and A Vincent unpublished observations) or from autoimmune thyroid tissue (Chazenbaik et al 1993; Guo et al 1999).

Since the dominant VH3-07 used by AMC-M2 was paired with so many unrelated light chains (both κ and λ), its VH probably makes the major contribution to binding specificity, as has frequently been observed with other antibodies (Guo et al 1999; Weigert and Potter 1977; Collet et al 1992; reviewed in Dorrington and Tanford 1970; Winter et al 19945). With AMC-M6, by contrast, the recurring usage of highly mutated and clonally related light chains in exclusive combination with the dominant VH3-21 suggests that they also have a major influence on specificity (as reviewed By Weigert and Potter 1977). There are long-established precedents for that in certain heritable restricted responses in mice to well defined epitopes (Weigert and Potter 1977), and also in human autoantibodies (Hoet et al 1999).

Importantly, the deduced ancestral sequence for each of the dominant families is already highly mutated (with 32, 44 and 25 shared differences from germline for AMC-M2 and AMC-M6 VH, and AMC-M6 Vκ respectively) indicating that the progenitor B cells had already undergone antigen-selection in GC before further refinement in the thymus. Almost all of the present sequences show multiple mutations, especially in the CDRs where replacement:silent ratios were often high. Of particular interest is the evidence of convergent mutations in the two patients' VH and especially in the recurrent ^{22}SRASET^{28} sequences in Vκ, suggesting that these Fabs may be recognizing a dominant fetal AChR epitope. Together with the abundant mutations, the high R:S ratios in the CDRs, and the branching patterns of their evolution, these results strongly suggest that each Fab is the end-product of successive stages of antigen-driven clonal proliferation in GC, as also found after *Haemophilus* vaccination in subjects with preexisting B cell memory (Hougs et al 1999). One can envisage that, over the preceding years, and four and two pregnancies respectively, the memory B cells that were initially generated could re-activate and/or re-enter GC for further rounds of mutational refinement. The striking patterns of oligoclonal evolution/diversification that we have observed in the fetal AChR-specific Fabs from both AMC mothers, are very reminiscent of those noted in a mouse model with spontaneous SLE, where a surprising variety of specificities stemmed from remarkably few ancestral B cells (Shlomchik et al 1987).

On the basis of these results, we suggest the initial immunization is by the fetus, generating a restricted group of mutated 'progenitor' memory cells, possibly in lymph nodes draining the uterus. Consequently, circulating antibodies are produced against fetal AChR; they attack rare myoid cells in the thymus that express this isoform (Schluep et al 1988), releasing antibody:AChR complexes that provoke local GC formation very efficiently (MacLennan 1994; Kunkl and Klaus 1981). The progenitor memory cells are attracted by the complexes trapped in these GC, and undergo further rounds of mutation and selection, culminating in the expanded populations of clonally related B cells and plasma cells that we have detected (Table 3, Fig 4). Independent evidence has already strongly implicated myoid

cells in GC formation in 36 EOMG thymic samples, including AMC-M2 and -M6 (I Roxanis, N Willcox, in preparation), as well as GCs in diversification of IgG antibodies in MG (Sims et al 2001).

Several autoimmune diseases show both a strong female bias and onset in early adulthood (eg SLE, thyroid disease, multiple sclerosis and EOMG); there is evidence for the importance of hormonal influences in the autoimmune response, as well as potential X chromosome contributions in these diseases (Whitacre 2001). Persisting fetal ↔ maternal microchimerism has also been invoked because of the recent findings of fetal or maternal RNA in scleroderma lesions (discussed in Maloney et al 1999; Nelson 2001). There are already well known examples of *allo*immunization of mothers, for example, by fetal erythrocytes or platelets (Blanchette, Johnson and Rand 2000). While it might be informative to look for allotypic variations in the AChR γ subunit, the AChR is remarkably *auto*immunogenic, even without adjuvant (Jermy, Beeson and Vincent 1993). Although the majority of EOMG female patients present before pregnancy, our results suggest that *auto*immunization by the fetus is another possible route to maternal MG, perhaps enhanced by presentation by semi-allogeneic (fetal) cells.

Methods

Clinical material. Patient material was obtained with informed consent and Ethical Committee approval. Sera from MG patients were collected in Professor John Newsom-Davis' clinic, before thymectomy or immunosuppression, and stored at –20°C. Antibodies to AChR were measured as previously described (Vincent et al 1995; Riemersma et al 1997). AMC-M2 and AMC-M6, described in (Riemersma et al 1997; Jacobson et al 1999a), had had four and two babies, respectively, with severe AMC (fatal in all but one), but the diagnosis of maternal MG was made only after the birth of the AMC babies. After therapeutic thymectomy and immunosuppressive therapies for their MG, and plasma exchange during pregnancy, both mothers have subsequently had successful pregnancies with minimally affected babies (unpublished observations). Thymus cell suspensions were prepared and

cryopreserved as in (Willcox, Newsom-Davis and Calder 1983). From both patients, these cells spontaneously produced high levels of anti-AChR antibodies in culture that were reduced by pokeweed mitogen, typical of plasma cell behavior (Willcox, Newsom-Davis and Calder 1983); these antibodies showed a strong preference for fetal AChR, and also inhibited its ion channel function (not shown), as did serum antibodies from AMC-M2 (Riemersma et al 1997) and AMC-M6.

Measurement of inhibition of AChR function in TE671 cell line. We measured AChR function in TE671 cells that express only fetal AChR, as previously described in (Riemersma et al 1997). Carbachol-induced $^{22}Na^{+}$ flux was measured over 1 minute, and the internalized $^{22}Na^{+}$ was counted on a Packard Autogamma counter. The inhibitory effect of the sera was tested by incubating the cells with 25 μl of serum in 500 μl Hepes Locke buffer (ie. 1:20) for 30 minutes. The results are expressed as % inhibition, with 0% being the cpm in Hepes Locke buffer alone and 100% inhibition being the cpm in cells tested in the presence 1 μg/ml of the antagonist α-bungarotoxin (α-BuTx). Those sera producing more than 50% inhibition were then retested at higher dilutions and the results are presented as % inhibition/μl of serum.

Combinatorial Ig gene library construction. The Fab library was made from cDNA after reverse-transcription from mRNA obtained from thymic cells (Farrar et al 1997). We used an anti-sense primer for an IgG1 CH1 sequence (almost identical to its IgG3 homolog (Kabat et al 1991)) to amplify VH chain cDNA by PCR in combination with a panel of sense oligonucleotide primers designed to include VH1-VH6 gene families: further panels amplified the Vκ or Vλ gene families, as described in (Farrar et al1997). The PCR products were ligated into Immunozap H or L bacteriophage vectors (Stratagene). Subsequently, the heavy and light chain DNAs were ligated into Immunozap to yield combinatorial libraries, as detailed in (Farrar et al 1997) and (Chazenbaik et al 1993).

Screening the library for AChR-binding Fabs. Positive clones were identified essentially as in (Farrar et al 1997). Muscle extracts from denervated muscles were labeled with 2 nM ^{125}I-α-BuTx (Amersham International, Amersham, UK; specific activity 2000 Ci/mmol) and used to screen the unamplified combinatorial library in XL1-Blue cells by filter-lift assays. Positive plaques were identified by autoradiography, cloned to homogeneity, and the heavy and light chain genes were sequenced in both directions by the dideoxy chain termination method (Sanger et al 1977). To obtain soluble Fabs, the XL1-Blue cells were induced with 1 mM isopropyl-thio-galacto-pyranoside (Sigma, St Louis, MO) overnight. The cells were then pelleted and freeze/thawed in 10 mM Tris buffer pH 8.0 containing protease inhibitors (see (Farrar et al 1997)). The suspension was sonicated and cleared by centrifugation, to leave a Fab-containing lysate.

Characterizing Fab reactivity with AChR. The lysates were used without further purification. They were incubated with AChR (either from muscle extracts, from TE671 cells or from a transfected subline that expresses predominantly adult AChR (Beeson et al 1996)), labeled with ^{125}I-α-BuTx. After 2 hours at 20˚C, carrier normal human serum was added plus a goat anti-human IgG (Lawrance Laboratories, Western Australia) which precipitated the Fab-AChR complexes efficiently. Competition with mAbs (Jacobson et al 1999b) was measured by preincubating the ^{125}I-α-BuTx-AChR with 50 μl of each Fab overnight at 4˚C and then adding excess (0.1 μl of ascites) of each mAb (see (Farrar et al 1997)). Sheep antibody to mouse IgG (which did not precipitate human Fabs) was then added to precipitate mAb-AChR complexes. All results were compared with precipitation in the presence of a control Fab that did not bind AChR, and expressed as the % inhibition by each Fab of the binding of the indicated mAb (see also (Whiting et al 1986)).

Bioinformatics. Sequences were compared with the human VBASE directory of immunoglobulin genes (Tomlinson et al 1997) using DNAPLOT (Müller, W. Institut für Genetik, Köln) to determine the best matching germline V-gene segments. Sequences with

the same gene rearrangement and common CDR3s were judged to be clonally related. The numbers of somatic mutations over the VH and Vκ regions were determined, and ratios of replacement to silent mutations (R:S ratio) were calculated for framework (FWR) and complementarity- determining regions (CDR). Amino acid numbering and FWR and CDR positions were previously defined by Kabat et al (1991). Genealogical trees were constructed for sets of related genes by analysis of shared and unshared mutations using phylogenetic analysis using parsimony (PAUP) (Swofford 1993). Independent genes were also compared for the occurrence of convergent mutations.

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Figure legends

Figure 1 Inhibition of fetal AChR function by sera from AMC-M2 and AMC-M6, from different groups of MG patients and healthy controls. Carbachol-induced $^{22}Na^{+}$ uptake into TE671 cells was measured after preincubation in each of the sera, with dilutions from 1:20 to 1:500. The inhibition of $^{22}Na^{+}$ uptake was expressed as a % of that obtained in the presence of α-BuTx, an irreversible antagonist of AChR function. Preliminary data were presented in Vincent et al 2001.

Figure 2 AChR binding by Fab clones. (a) Example of immunoblot plaque lifts to detect Fab clones binding ^{125}I-α-BuTx-AChR extracted from ischemic human leg muscle. (b) Immunoprecipitation of ^{125}I-α-BuTx-AChR by representative cloned Fabs from AMC-M2 and (c) from AMC-M6.

Figure 3 Specificity of cloned Fabs. (a) Reactivity of AMC-M2 and AMC-M6 serum antibodies and representative Fabs with adult and fetal AChR. The AChRs were obtained from TE671 cells (fetal AChR) and from TE671 cells transfected with excess of the AChR ε subunit (providing approximately 90% adult AChR and 10% fetal AChR). (b) Competition of AMC-M2 serum and Fabs with mAbs directed against the indicated AChR subunit. All of the Fabs inhibited the binding of mAbs specific for the γ subunit (c) Competition of AMC-M6 serum and Fabs with mAbs, as above. All but one of the Fabs inhibited binding of mAbs specific for the γ subunit, the other only inhibited binding to a mAb specific for the β subunit. The characterization of the mAbs is detailed in Jacobson et al 1999b.

Figure 4 Evolution of VH and VK clones from AMC-M6.

Sequence alignments and genealogical trees for (a) AMC-M6 VH3-21 heavy chain and (b) VK1 O2/12 kappa chain genes. The Fab nucleotide sequences were aligned with the best-matching germline genes. Only codons at which mutations occur are shown, and any amino acid changes are indicated above. The most parsimonious genealogical trees were

determined on the basis of shared and unshared mutations using the PAUP program (Swofford 1993). Fab names are shown in the circles with H referring to the heavy chain and K referring to the kappa chain. Dotted circles refer to hypothetical intermediates. Numbers beside arrows show the minimum number of substitutions required at each step (which may include multiple changes at a single nucleotide position).