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Acetylcholine receptor antibody characteristics in myasthenia gravis. I. Patients with generalized myasthenia or disease restricted to ocular muscles

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SUMMARY

We have investigated anti-acetylcholine receptor (AChR) characteristics in three forms of generalized myasthenia gravis, which are distinguishable by their thymic pathology, age of onset, sex incidence and HLA antigen associations, and in restricted ocular myasthenia. Light chain, IgG subclass, avidity for denervated human AChR and reaction with various human and mammalian AChR preparations were examined in 9–12 patients from each group. Characteristics varied between individuals in each group but no significant differences were found in mean values between the three groups with generalized myasthenia. When antibody characteristics in ocular myasthenia were compared with those in generalized disease, however, differences were found particularly in relative reactivity with denervated, normal and ocular human AChR. The nature of the humoral response in ocular myasthenia thus differs from that in generalized myasthenia. The similarity in the humoral response between the three groups of generalized myasthenia suggests, on the other hand, that the distinguishing clinical features may reflect differing susceptibilities to diverse precipitating mechanisms.

INTRODUCTION

Myasthenia gravis (MG) is a disease of the skeletal neuromuscular junction in which weakness and fatiguability result from a decrease in the number of post-synaptic acetylcholine receptors (AChR). Anti-AChR antibodies are present in 85–90% of patients' sera (Lindstrom *et al.*, 1976; Compston *et al.*, 1980) and there is good evidence to implicate them in the loss of functional AChRs although the level of antibody does not correlate with the severity of the disease (Vincent, 1980). This observation could be explained by heterogeneity of anti-AChR which appears to vary in its characteristics between individuals (Lefvert & Bergström, 1978; Lindstrom, Campbell & Nave, 1978; Vincent & Newsom-Davis, 1979).

We recently analysed the clinical and immunological features of 68 patients with MG (Compston *et al.*, 1980). Patients could be divided into three groups based on the age of presentation and thymic pathology: (a) those with thymoma who presented mostly between 30 and 50 years of age and showed no clear HLA association; (b) those presenting under 40 years of age who tended to be female, have hyperplasia of the thymus and possess HLA B8 and (c) those presenting over 40 years of age who tended to be male and HLA B7.

In addition we have found that patients with symptoms restricted to ocular muscle have

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significantly lower anti-AChR titres than those with generalized disease (Compston *et al.*, 1980), but the nine patients in this category showed no obvious HLA associations (Newsom-Davis, Vincent & Compston, unpublished results). The question arose as to whether the characteristics of anti-AChR antibody differed between these groups of patients and might relate to the HLA associations found.

MATERIALS AND METHODS

Patients. Clinical details of the patients are given in Table 1.

The first serum sample available was taken from 11–12 representative patients from each of the three main groups defined above and from nine further patients who had had symptoms essentially restricted to the extraocular muscles for more than 2 years and had sufficient anti-AChR titres (over 0·8 nmoles/l) to allow characterization.

Acetylcholine receptor preparation. Alpha-bungarotoxin (α-BuTx) (Miami Serpentarium) was iodinated to a specific activity of $20–50 \times 10^6$ c.p.m./μg (Compston *et al.*, 1980). Several different muscle AChR extracts were used:

(1) Amputated 'denervated' human calf muscle, from a patient with diabetes mellitus and peripheral neuropathy, was stored at −70°C, and contained 4 pmoles/g of 'd-AChR' most of which would be the extra-junctional form which appears after denervation.

(2) Bilateral 'normal' calf muscles, taken 3 hr post mortem from a patient dying from cerebral disease, contained 0·8 pmoles/g 'n-AChR' which would be largely normal junctional (end-plate) AChR.

(3) Extraocular muscles, obtained similarly from the patient in (2), contained 2 pmoles/g 'oc-AChR'.

AChR was also extracted from cultured fetal human muscle, rat denervated leg muscle and a mouse non-fusing cell line (BC3H1, kindly donated by Dr S. Bevan).

The muscle was homogenized and extracted with an equal volume of 1% Triton X-100 and AChR was assayed by ^{125}I-α-BuTx binding at 2·5 nM using DEAE filter discs (Whatman DE 81) (Compston *et al.*, 1980).

Anti-AChR assays. All assays were done at 0·1 nM AChR (10 fmoles) labelled with ^{125}I-α-BuTx (approximately 80% saturation) and diluted in 20 mM pH 7·4/0·1% Triton X-100 (PTX buffer), unless otherwise stated. Sera were titrated against d-AChR and all results related to these. The amount of serum used precipitated approximately 40% of the AChR at 0·1 nM.

The anti-AChR assays were performed on all sera simultaneously using 0·025–2·5 μl of MG serum, control serum as carrier if necessary, and 10–25 μl anti-IgG (Fc) (Seward Laboratories Ltd) (Compston *et al.*, 1980). In each set three negative and one positive control sera were run. In assays for low antibody (e.g. ocular MG sera) results were confirmed by incubating sera (2·5–5 μl) with both ^{125}I-α-BuTx labelled AChR, and AChR which had been saturated with non-radioactive α-BuTx before ^{125}I-α-BuTx addition and the difference in c.p.m. taken.

Cross-reactivity was tested by incubating serum dilutions with 10 fmoles of ^{125}I-α-BuTx labelled d-AChR, n-AChR, oc-AChR and AChR from fetal human myotube cultures, denervated rat leg muscle and the mouse cell line.

Proportion of light chains and IgG subclasses. The AChR–antibody complexes were precipitated with sheep anti-sera specific for light chains and IgG subclasses (Seward Laboratories Ltd) aided by 3% polyethylene glycol (PEG) as described in detail elsewhere (Vincent & Bilkhu, 1982). Anti-sera and normal sheep serum (NSS) were first treated with 3% PEG 6000 (BDH Chemicals Ltd) to precipitate aggregated proteins. MG sera (~40 fmoles anti-AChR) were incubated with 100 fmoles ^{125}I-α-BuTx labelled d-AChR for 4 hr in 250 μl. Separate 25 μl aliquots + 25 μl PTX were incubated overnight at 4°C with 50 μl of NSS, 50 μl anti-IgG (Fc) or 50 μl of each anti-sera. Fifty microlitres 6% PEG was added for 1–2 hr before spinning for 15 min at 4000 rpm. The pellets were washed twice for 1 min in PTX. Subclass results are expressed as percentage of precipitation with anti-IgG (Fc) after subtraction of NSS controls. The κ light chains are expressed as % of the sum of precipitation with anti-κ and anti-λ.

Inhibition of α-BuTx binding. Fifty femtomoles of d- and n-AChR were incubated overnight with ~500 fmoles of anti-AChR in 300 μl of PTX. The binding of ^{125}I-α-BuTx (5nM) was measured in duplicate by the filter disc assay. The c.p.m. inhibited compared to control sera incubations was expressed as a percentage of the c.p.m. precipitated by each serum based on results at limiting dilutions.

Anti-AChR avidity. A constant amount of each serum (~4 fmoles) was incubated for 4 hr at room temperature with 100 μl d-AChR at 0·1 and 0·4 nM and the results expressed as a ratio. In 20 cases anti-AChR (3–8 fmoles) was incubated with d-AChR between 0·03 and 0·5nM, the samples left for 8–10 hr at room temperature and Scatchard plots drawn. Similar experiments were performed with mouse AChR.

Statistics. Differences between the groups were assessed by Student's *t*-test. Where the variances of the groups differed, the significance of the *t*-test was tested by the method of Cochrane & Cox (see Downie & Heath, 1974). The Pearson correlation coefficient was used for data within groups.

RESULTS

Patients with generalized myasthenia

Anti-AChR characteristics were investigated in three clinical groups of patients (thymoma; non-thymoma, onset <40 years; non-thymoma, onset >40 years) whose main features are summarized in Table 1. Ocular cases have been excluded from these three groups and are considered separately later.

κ and λ light chains

Anti-AChR titres, at 0·4 nM d-AChR, are given in Table 2. In each group a high proportion of the anti-AChR was κ light chain but there was some variation between the groups (see Fig. 1a). In six of the 35 patients more than 95% of the anti-AChR was precipitated by anti-κ. In contrast, only two patients had anti-AChR which was more than 60% λ. Differences between the groups were not significant (Table 2).

IgG subclasses

The results of IgG subclass 1 and 2 determinations were not quantitative, notably in patients with

Table 1. Main clinical characteristics of patients

	Group			
	1	2	3	4
Definition	Thymoma +ve	Thymoma −ve onset <40y	Thymoma −ve onset >40y	Ocular only
Number tested	12	11	12	9
M:F	7:5	3:8	8:4	8:1
HLA, number +ve, B7, B8, Dr2, Dr3, Dr7	6, 1, 4, 3, 6	0, 10, 2, 9, 0	9, 2, 7, 0, 3	Insufficient data
At time of sampling				
Duration of symptoms (years) (range)	9±11 (1–40)	6±4·5 (1–15)	5±3·9 (1–11)	6±6 (2–13)
Clinical classification,* number in each group R, I, IIa, IIb, III, IV	0, 0, 1, 3, 1, 7	0, 1, 3, 3, 1, 3	2, 2, 5, 2, 1, 0	0, 9, 0, 0, 0, 0
Number thymectomized	12	11	2	0
Number on immunosuppression	9	5	7	5

* Based on Osserman & Jenkins (1971).

Table 2. Results in main groups of patients

	1	2	3
Number of patients*	12	11	12
Mean anti-AChR titre	98·5±78·9	74·6±71·9	37·8±55·8
% κ light chain	83±14	71±24	79±25
% IgG1 (number tested)	29±12 (8)	26±18 (6)	17±9 (2)
% IgG2	40±17 (8)	25±14 (6)	28±8 (2)
% IgG3	9·1±8·6	16±20	12±10
% IgG4	10±9·1 (8)	9·2±7·4 (6)	11±1·0 (2)
% anti-α-BuTx site:			
denervated AChR	3·7±5·6	3·4±4·0	3·8±1·8
normal AChR	1·4±2·2	1·7±2·2	3·5±3·1
% anti-normal AChR	82±28	98±47	91±19
% anti-ocular AChR	55±23 (7)	47±17 (7)	55±22 (7)
% anti-rat AChR	19±10	20±21	17±20
% anti-mouse AChR	16±9·1	11±7·5	11±10
Anti-AChR avidity:			
(AChR 0·1 nM/AChR 0·4 nM)	0·61±0·1	0·58±0·1	0·63±0·1
K_D from Scatchard plots ($\times 10^{-12}$ M)	26·3±15·8 (6)	25·1±11·8 (8)	21·5±10·5 (6)

All results are mean±s.d. * When less patients tested, numbers given in parentheses.

low anti-AChR titres. The contribution of subclass 1 was probably underestimated and subclass 2 may have been over-estimated since we have found that anti-IgG2 cross-reacts with IgG1 (Vincent & Bilkhu, 1982). Table 2 gives the results where the precipitation with anti-subclass specific anti-sera added up to ≥60% of the total anti-AChR present. Most patients have anti-AChR distributed mainly in IgG1 and IgG2, but the ratio of IgG1 to IgG2 anti-AChR varied (Vincent & Bilkhu, 1982). One patient from Group 2 had more than 60% IgG3 (Fig. 1b). There were no significant differences in the subclass results between the three groups of patients.

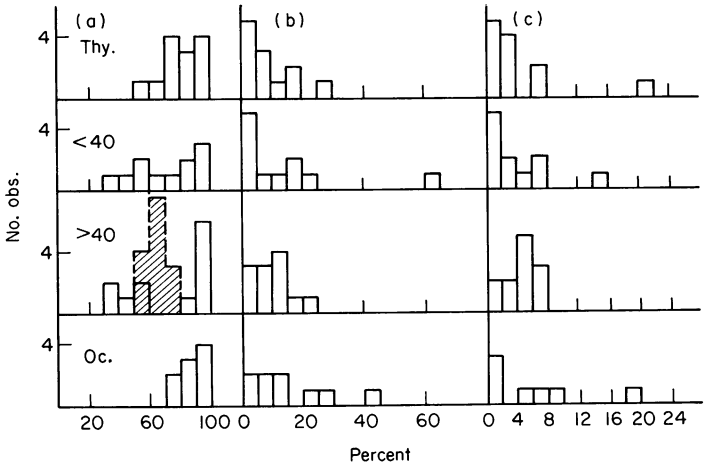

Fig. 1. Antibody characteristics in different forms of generalized and ocular MG presented as histograms in three vertical columns divided horizontally into the four groups of patients. The dashed lines and hatched area represent the distribution of % κ values in normal IgG. All results are % of total anti-d-AChR. (a = κ; b = IgG3; c = anti-α-BuTx site).

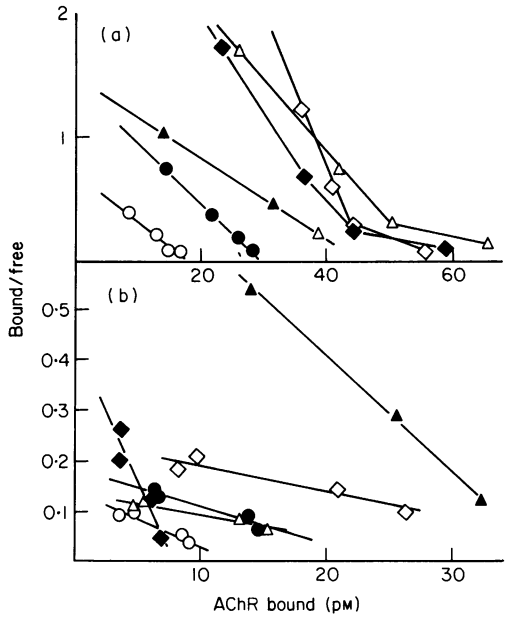

Fig. 2. Avidity of anti-AChR for human (a) and mouse (b) AChR. (a) Scatchard plots of antibodies from six different patients, including two thymoma cases (♦ ◊), two non-thymoma cases (▲ △) and two ocular patients (● ○). The avidity of the antibody is indicated by the slopes of the lines (drawn by eye). D-AChR concentration varied between 0·03 and 0·5 nM, incubation time 10 hr. (b) Binding to AChR extracted from the mouse cell line. Serum varied between one and five times that used in (a). Cross-reactivity varied between 4 and 87% at 0·1 nM.

Inhibition of α-BuTx binding

The extent to which sera inhibited binding to d-AChR was variable but in most cases anti-α-BuTx binding site antibodies represented less than 5% of the total anti-AChR present (Fig. 1c; Table 2).

Anti-AChR avidity

All 35 sera were assayed at two d-AChR concentrations. The mean ratio of the counts precipitated (range 0·38–0·88) for each group (Table 2) was very similar. The mean values for the K_{Ds} observed from Scatchard plots of more detailed analysis (Fig. 2a) also do not differ significantly. With some sera there was a suggestion of a lower avidity binding constant at high d-AChR.

Cross-reactivity

Cross-reactivity of anti-AChR with different AChR preparations varied within each group. When compared with d-AChR, all patients reacted similarly with fetal myotube AChR (not shown), and many reacted less well and variably with n-AChR. However, one patient (in Group 2) reacted considerably better (2·2-fold) with n-AChR (Fig. 3a).

Cross-reactivity with xenogeneic AChR (0·1 nM) was also variable but the differences between the groups were insignificant. In two sera the avidity for mouse-AChR was similar to that for human d-AChR (Fig. 2b) with K_{Ds} in the range $20–40 \times 10^{-12}$ M, but in four sera, which had shown $< 5\%$ cross-reactivity at 0·1 nM, the avidity for mouse AChR was lower with K_{Ds} of $80–120 \times 10^{-12}$ M.

Correlations between HLA and different antibody characteristics

In spite of the variability within each group there were no significant differences in antibody characteristics between the three main groups (Table 2). Some investigations were also performed

independently on seven patients from each group who showed the most typical age, sex and HLA characteristics but there were no significant differences between them in % κ light chain and anti-AChR avidity (not shown). As previously reported (Compston *et al.*, 1980), patients with generalized MG tended to react less with oc-AChR than with d-AChR (Fig. 3b), and this did not differ between the groups (Table 2).

Patients with HLA DR7, irrespective of their group, had a higher proportion of κ light chains ($88 \pm 11{\cdot}6$ s.d., $n=9$) than those without this antigen ($75{\cdot}1 \pm 22{\cdot}8$ s.d., $n=26$) ($P<0{\cdot}05$). There was a correlation ($r=0{\cdot}55$, $P<0{\cdot}001$, $n=35$) between % λ light chain and % IgG3. No significant correlation was observed between reactivity with n- or oc-AChR relative to d-AChR ($r=-0{\cdot}20$), although there was a correlation between reactivity with d-AChR and that with human fetal myotube AChR ($r=0{\cdot}96$, $P<0{\cdot}001$, $n=35$), the extrajunctional form of receptor which is present before innervation. Neither the antibody avidity nor the effective antibody titre (i.e. titre/K_D) showed any clear relationship with disease severity.

Ocular myasthenia gravis

Anti-AChR characteristics were investigated in nine patients with ocular MG and the results compared with the pooled data from the 35 generalized MG cases. The anti-AChR titre in ocular patients was much lower ($P<0{\cdot}001$) than that of the 35 patients with generalized disease. κ and λ light chains and IgG3 were investigated using an equal mixture of n-AChR and d-AChR because ocular sera tend to react better with n-AChR. Anti-AChR ranged from 77 to 100% κ, with a skewed distribution (Fig. 1). The mean values for % κ and % IgG3 were significantly different from those of patients with generalized MG (Table 3).

Ocular MG sera showed higher inhibition of α-BuTx binding to the AChR as a proportion of their relatively much lower anti-AChR titres, but the differences from the generalized group were not significant.

The avidity of the antibody was not apparently different from that in patients with generalized myasthenia (Table 3 & Fig. 2a).

Five out of the nine ocular patients reacted better with n- than with d-AChR (Fig. 3), and these patients also tended to react better with oc-AChR as previously reported (Compston *et al.*, 1980). In ocular cases there was a positive correlation between anti-n and anti-oc reactivity ($r=0{\cdot}53$, $n=8$), but this did not reach significance.

Only one of seven patients tested against rat or mouse AChR had more than 5% cross-reactivity when short incubation times were used (Table 3). However, at longer incubation times and at higher

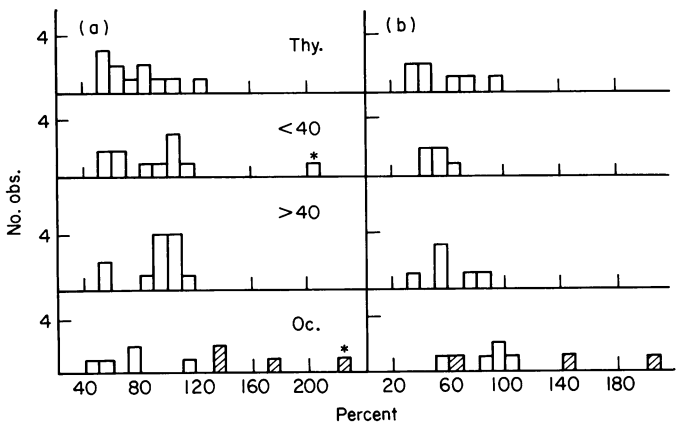

Fig. 3. Antibody binding to n- (a) and oc-AChR (b) compared with d-AChR in the four groups of patients as in Fig. 1. The results from the main groups were those of patients with the typical HLA characteristics. Two of the sera (*) with high reactivity for n-AChR were not tested against oc-AChR. The hatched columns represent results from patients with pure ocular disease of more than 2 years' duration who had not had immunosuppression.

Table 3. Comparison between generalized MG and ocular MG

	Groups 1, 2 and 3 pooled	Ocular only
Number of patients	35	7–9*
Anti-AChR titre	71·8 ± 72·4	3·1 ± 1·8 (9)§
% κ light chain	78·5 ± 21·1	91·4 ± 6·7 (9)†
% IgG3	9·8 ± 12·7	23·1 ± 10·3 (8)†
% anti-α-BuTx site:		
denervated AChR	3·7 ± 3·9	5·7 ± 6·5 (7)
normal AChR	2·3 ± 2·7	5·5 ± 6·6 (7)
% anti-normal AChR	90·1 ± 32·4	117·8 ± 68·5 (9)
% anti-ocular AChR	54·0 ± 20·6 (21)	107·1 ± 47·0 (8)†
% anti-rat AChR	18·6 ± 17·3	4·3 ± 11·3 (7)†
% anti-mouse AChR	11·8 ± 9·3	1·6 ± 4·2 (7)§
Anti-AChR avidity:		
(AChR 0·1 nM/AChR 0·4 nM)	0·61 ± 0·1	0·72 ± 0·2 (7)
K_D from Scatchard ($\times 10^{-12}$ M)	23·5 ± 12·4 (20)	32·6 ± 11·9 (5)

* Not all data was obtained from ocular patients, numbers in parentheses refer to number tested.

† $P < 0.05$, ‡ $P < 0.01$, § $P < 0.001$. All results are mean ± s.d.

AChR concentrations (up to 1 nM mouse AChR), cross-reactivity could be detected (e.g. see Fig. 2b) although, as in most cases of generalized MG, the avidity of the cross-reaction was low.

DISCUSSION

Our results provide further evidence that anti-AChR is usually polyclonal and heterogeneous in its reactivity with different preparations of receptor and in its ability to inhibit α-BuTx binding to AChR (compare Lefvert & Bergström, 1978; Lindstrom *et al.*, 1978; Vincent & Newsom-Davis, 1979). This heterogeneity would be consistent with the poor correlation between anti-AChR and clinical state across individuals. However, it appears that the variable cross-reactivity of anti-AChR with different AChR preparations may be due not only to the absence of antibody to different antigenic determinants but also to the relatively poor avidity of the antibodies for these determinants. Whether relative avidity also plays a part in the apparent cross-reactivity with n- or oc-AChR remains to be seen.

In generalized MG sera the proportion of κ light chain anti-AChR was significantly greater ($P < 0.001$) than that in normal serum (Eickhoff & Heipertz, 1977; see dashed line in Fig. 1a), and in some patients anti-AChR was >95% κ light chain. The polyclonality of the antibody in most patients is similar to that found in thyroglobulin antibodies (Fahey & Goodman, 1964) but contrasts with, for instance, chronic cold agglutinins which are monoclonal κ light chain (Harboe, 1964).

Some human antibodies are restricted in subclass. Rheumatoid factor, for example, is found in subclasses IgG1 and IgG3 and anti-factors VII antibodies are almost exclusively IgG4 (Natvig & Kunkel, 1968). We have found that the relative proportions of the anti-AChR subclasses were similar to the proportions of ^{125}I-IgG precipitated at saturation by the various anti-sera, and therefore probably represent a distribution between the subclasses according to their total amount (Vincent & Bilkhu, 1982), similar to results with thyroglobulin antibodies (Hay & Torrigiani 1974). The low proportion of subclass 3 anti-AChR found here agrees with results reported by Tindall (1981) but contrasts with a much higher proportion reported by Lefvert & Bergström (1978). They used protein A Sepharose to adsorb subclasses IgG1, 2 and 4 and obtained >50% recovery of anti-AChR in the effluent. When we performed similar experiments, the mean recovery of

anti-AChR was only 11%, which is close to the value for IgG3 in normal serum (Vincent & Bilkhu, 1982).

We have demonstrated a population of very high avidity antibody (mean K_D $23{\cdot}5 \pm 2.89$ (s.e.m.) $\times 10^{-12}$ M, $n = 20$) in each patient. Lower avidity antibodies probably co-exist as suggested by some of the data at higher AChR concentrations (Fig. 2), and may contribute to the clinical state by binding to the AChR. Nevertheless, the results presented here contrast to those in patients with penicillamine-associated MG (Vincent & Newsom-Davis, 1982) where the anti-AChR avidity is generally lower.

The lack of difference between the three main groups as regards any of the anti-AChR characteristics examined suggests that the nature of the humoral immune response is the same in all three main groups of patients with idiopathic generalized MG. However, the duration of the disease in the patients examined was extremely variable and in a further study (Vincent & Newsom-Davis, 1982) we have found a trend towards changing antibody characteristics during the course of the disease which might contribute to the variability in characteristics observed within each group. Variability in anti-AChR characteristics could also depend on immunogenetic influences, and we found that patients with DR7 tended to have high κ light chain anti-AChR. However no other anti-AChR characteristics, apart from anti-AChR titre (Compston *et al.*, 1980), could be shown to correlate significantly with the HLA associations found in the three main groups of patients. These findings suggest that although genetic factors may influence overall antibody levels, the HLA associations found in the different groups relate to the precipitating factors involved in initiation of the disease rather than to the nature of the humoral response itself.

Ocular MG might be considered on clinical grounds to be a separate form of the disease because of the highly restricted symptoms, male preponderance, relatively poor response to anti-cholinesterase treatment, and good response to steroids (Fischer & Schwartzman, 1976). Moreover, the anti-AChR titre is usually very low and many typical ocular cases give negative results (Lindstrom *et al.*, 1976; Compston *et al.*, 1980). However, our findings suggest that antibody characteristics as well as titre may contribute to the clinical expression of the disease. As a group ocular sera react better with oc- or n-AChR compared to d-AChR than do generalized MG sera and this is best exemplified by the four cases in whom symptoms had been entirely confined to the ocular muscles for 2–13 years in the absence of immunosuppressive treatment (Fig. 3).

Our results indicate that n-AChR shares more antigenic determinants with oc-AChR than with d-AChR. Our previous finding (Vincent & Newsom-Davis, 1979) that MG sera reacted similarly to denervated and putatively 'normal' muscle AChR suggests that the latter, obtained from an ischaemic limb, may also have been partly denervated. Antigenic differences have also been shown between normal and denervated rat (Weinberg & Hall, 1979) and human AChR (Lefvert, 1981), but Lefvert did not report any sera which reacted preferentially with normal AChR.

Wekerle *et al.* (1981) have proposed that sensitization to AChR takes place in the thymus owing to the (?abnormal) expression of AChR on non-innervated, 'myoid' cells. If this is the case one might expect that the humoral response would preferentially recognize extrajunctional d-AChR. This seems to be the case in many patients with generalized disease (Fig. 3). The fact that several sera from ocular MG patients react better with n-AChR suggests that the nature of the autoantigen, and therefore possibly the site of the autosensitization, may differ. Ocular sera, however, tend to react similarly with n- and oc-AChR and the susceptibility of ocular muscles to clinical weakness may therefore be partly due to other factors such as a lowered safety factor for neuromuscular transmission (Vincent, 1980).

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