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Fine antigenic specificities of antibodies in sera from patients with D-penicillamine-induced myasthenia gravis

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SUMMARY

A small fraction of patients with rheumatoid arthritis and other diseases on D-penicillamine treatment may develop antibodies against the acetylcholine receptor (AChR) and symptoms of myasthenia gravis (MG). The mechanism leading to this phenomenon is not known. We have studied the fine antigenic specificities of the anti-AChR antibodies in 19 D-penicillamine-induced MG (pen-MG) patients and compared them with those of antibodies from 204 idiopathic MG patients (the data for 122 obtained from earlier experiments). Antigenic specificities of the circulating antibodies were determined by the capacity of monoclonal antibodies (MoAbs), against certain determinants on the AChR, to inhibit binding of the serum antibodies to the AChR. Monoclonal antibodies against α, β and γ subunits were used. The anti-AChR antibody patterns of pen-MG patients were very similar to those of idiopathic MG patients. Antibodies to the main immunogenic region, which is located on the extracellular surface of the α-subunit, were the predominant group. The variations of antibody specificities in serial sera collected from individual patients at different times were usually small, as were those of idiopathic MG. These results strongly suggest that the antibody repertoire in the sera of idiopathic and pen-MG patients is very similar.

Keywords Myasthenia gravis D-penicillamine monoclonal antibodies acetylcholine receptor anti-sera

INTRODUCTION

Idiopathic myasthenia gravis (MG) is considered to be caused by the spontaneous development of antibodies against the muscle nicotinic acetylcholine receptor (AChR) (Drachman *et al.*, 1987; Lindstrom, 1985). These antibodies result in loss of AChRs and also directly block the function of the remaining molecules, thereby causing a defect in neuromuscular transmission.

A small percentage of patients with rheumatoid arthritis or Wilson's disease who receive D-penicillamine develop symptoms of MG (Bucknall, 1977) and their sera contain anti-AChR antibodies (Russell & Lindstrom, 1978; Vincent & Newsom-Davis, 1982). Both the symptoms and the antibodies remit within a few months after drug cessation (Albers *et al.*, 1980; D'Anglejan *et al.*, 1985). Experimental induction of anti-AChR antibodies by D-penicillamine in animals and in cell cultures has not proved very successful (Burres *et al.*, 1981; Scadding, Calder & Newsom-Davis, 1983). It is likely that D-penicillamine induces MG in genetically pre-disposed individuals by changing their immunomodulation (Oosterhius, 1984). The drug may mimic the unknown trigger of idiopathic MG. Studying the similarities or differences between the end products of the two disorders (i.e. the anti-AChR antibodies) conclusions could be drawn regarding the pathogenetic mechanisms of autoreactivity in MG. Immunoglobulin characteristics of the D-penicillamine-induced MG (pen-MG) antibodies and their affinity for human and mammalian AChR have been reported as being similar to those of idiopathic MG patients with recent disease onset, although differences were found in the avidity for the antigen and the light chain composition when compared with the overall idiopathic MG population (Vincent & Newsom-Davis, 1982). Sera from pen-MG patients have the capacity to inhibit α-bungarotoxin binding to the AChR in a similar manner to idiopathic MG sera (Vincent & Newsom-Davis, 1982; Kuncl *et al.*, 1986).

Competition experiments between anti-AChR MoAbs and sera either from MG patients or from immunized rats showed that about two-thirds of the human anti-AChR antibodies are directed towards the main immunogenic region (MIR) (Tzartos *et al.*, 1981, 1982; Tzartos, 1988). The MIR is located on the extracellular surface of the α-subunit (Tzartos & Lindstrom, 1980; Sargent *et al.*, 1984), between amino acid residues 67–76

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Table 1. Characteristics of the MoAbs used as protectors of human AChR*

MoAb No.	Ig class	Binding to AChR from† H	M	T	E	Subunit, epitope and region specificity	Transfer MG to rats	Increase AChR degradation
42	G2a	+	+	++	++	α67–76, MIR	++	++
35	G1	++	++	++	++	α6–85, MIR	++	++
195	G1	++	++	−	ND	α, MIR	ND	ND
202	G1	++	++	−	ND	α, MIR	ND	ND
64	G2a	+	++	+	±	α, not MIR	−	−
73	G1	+	++	−	−	β, near MIR‡	−	+
66	G2a	+	++	−	−	γ, near MIR‡	−	+
155	G2a	+	+	++	+	α371–378, cytopl.	−	−
124	G1	+	+	++	++	β, cytopl.	−	−

* From Tzartos *et al.* (1981; 1983; 1985; 1986; 1987; 1988); Barkas *et al.* (1987); Ratnam *et al.* (1986) and Tzartos & Starzinski-Powitz (1986).

† AChR from human muscles (H), other mammalian muscles (M), Torpedo (T) or Electrophorus (E) electric organs. The source of the immunogen is indicated as underlined. Immunogen for MoAbs 64, 73 and 66 was fetal calf AChR. ++, +, ±, −; strong, medium, weak and no binding to the corresponding AChR.

‡ MoAbs 73 and 66 partially compete with the anti-MIR MoAb 35 (40% and 25% inhibition of MoAb 35 binding to fetal calf AChR by MoAbs 73 and 66, respectively). However, they do not compete with each other (Tzartos *et al.*, 1986 and unpublished).

ND, Not determined.

(Tzartos *et al.*, 1988). In this study, the fine antigenic specificities of the anti-AChR antibodies in sera from pen-MG patients have been determined using similar competition experiments. No significant differences were observed between pen-MG and idiopathic MG, suggesting that pen-MG is a valuable model for the study of MG.

MATERIALS AND METHODS

AChR and α-bungarotoxin

Crude muscle extracts derived from amputated human legs were prepared for use as ^{125}I-α-bungarotoxin labelled antigen (Lindstrom, Einarson & Tzartos, 1981). Alpha-bungarotoxin (from Sigma) was labelled with ^{125}I by the Chloramine-T method.

MoAbs

Preparation and characterization of the MoAbs used has been described earlier (Tzartos *et al.*, 1981; 1983; 1986) and is summarized in Table 1. All MoAbs were derived from rats immunized with intact or SDS-denatured AChR.

Human sera

Pen-MG sera were collected from 15 French (D'Anglejan *et al.*, 1985) and four Greek patients (nos, 3, 7, 11 and 17). All patients were treated for rheumatoid arthritis except for patient no. 15 who was treated for cystinic lithiasis. For comparison purposes, 82 sera from idiopathic MG patients (20 from France and 62 from Greece) of similar titre to the pen-MG antibody titres were used. Data from earlier experiments on 122 idiopathic MG patients Tzartos *et al.*, 1982; 1985; Tzartos & Morel, unpublished) were also compared. Antibody titres were determined according to Lindstrom *et al.* (1981). Pools of sera were prepared by mixing equal quantities of anti-AChR antibodies from each serum rather then equal serum volumes.

Competition between MoAbs and MG sera for human AChR binding

This was performed according to Tzartos *et al.* (1982; 1985). Samples of human muscle extracts in PBS plus 0·5% Triton X-100 containing 2 nM AChR with 10 nM ^{125}I-α-bungarotoxin were incubated for 3 h with a large excess of a specific MoAb (> 35 times the concentration of the AChR, taking into account their titres for human muscle AChR), or with control MoAb 25. The mixtures were dispensed into flexible, 96-well plates (20 μl per well, containing 5 fmol AChR) to which were added 20 μl per well PBS, 0·5% Triton X-100, containing MG serum with about 4 fmol anti-AChR antibody (or normal human serum, NHS, for the controls), supplemented with NHS to give a total serum volume of 0·5 μl (or 2 μl for very low titre sera). After 3 h pretreated rabbit anti-human gamma-globulin serum was added, incubated for 1 h, centrifuged and washed. Pretreatment of the rabbit serum comprised overnight incubation with 5% normal rat serum followed by centrifugation in order to eliminate antibodies cross-reactive with rat immunoglobulins. All incubations were performed at 4°C. The radioactivity present in the individual wells was counted. After subtraction of the background counts (radioactivity precipitated with NHS; 2–5% of the positive control), the percentage inhibition of binding by the MoAbs was estimated by the equation:

$$\% \text{ inhibition of binding} = \frac{\text{ct/min (MG.MoAb-25)} - \text{ct/min (MG·MoAb-i)}}{\text{ct/min (MG.Mob25)}} \times 100$$

Where ct/min (MG·MoAb-25) and ct/min (MG·MoAb-i) is the radioactivity precipitated by the MG serum in the presence of control MoAb 25 or human AChR-specific inhibiting MoAb, respectively. This inhibition (or protection against binding of serum antibodies) is assumed to represent the percentage of

Table 2. Some characteristics of the pen-MG patients and their sera

Patient no.	Sex	Age (yrs)	Duration of MG symptoms at sampling	Duration of pen. treatment (months)	Serum anti-AChR titre (nM)
1	F	70	1·5 months	18	1·7
2	F	75	24 months	18	2·4
3*	F	54	2 days	5	2·6
4	F	83	6 months	6	3·5
5	F	50	ND	ND	4·5
6	M	31	1 month	3	5·1
7*	F	46	10 days	4	8
8	F	59	4 months	16	8
9	M	56	5 months	60	12
10	F	45	12 months	ND	23
11*	F	ND	ND	ND	24
12	F	60	12 months	36	25
13	F	61	ND	12	32
14	F	59	1 month	60	33
15	M	32	24 months	24	36
16	M	56	20 days	30	39
17*	F	ND	ND	ND	41
18	F	55	ND	ND	42
19	F	30	ND	ND	115

* Greek patients. All others were from France.

antibodies capable of recognizing the MoAb-bound region of the AChR. Sera from both pen-MG and idiopathic MG patients were tested simultaneously in each individual experiment.

RESULTS

The Sera and the Technique Used

Table 2 shows some of the characteristics of the pen-MG patients. Patients nos 2, 10, 12 and 15 developed autonomous MG, subsequent to D-penicillamine treatment.

For the determination of the fine antigenic specificities of the human antibodies, human AChR labelled with ^{125}I-α-bungarotoxin was preincubated with anti-AChR MoAbs, followed by incubation with human serum. The fraction of human antibodies otherwise capable of recognizing the antigenic determinants, which in this instance were covered by the protecting rat MoAb, would not be able to bind. This fraction is representative of the percentage of human antibody binding. The regions were classified according to the number of the protecting MoAb, e.g. region-73.

Occurrence of Anti-MIR Antibodies

The occurrence of anti-MIR MG antibodies was investigated using four anti-MIR MoAbs (Fig. 1, lower bars). Usually, high proportions of human antibodies were inhibited by the anti-MIR MoAbs, especially by MoAbs 195 and 202 (Fig. 1). The average data in Fig. 2 show that 61–75% of the pen-MG antibodies were inhibited by each anti-MIR MoAb, i.e. similar to the inhibition values observed for idiopathic MG antibodies. In most cases, the four anti-MIR MoAbs used significantly inhibited different amounts of antibodies from a given serum, thus allowing for their division into two overlapping subgroups: MoAbs 42, 35 and MoAbs 195, 202 (note patient no. 10).

Occurrence of Antibodies in the Non-MIR Regions

Three of the non-anti-MIR MoAbs used (MoAbs 64, 73 and 66), exhibited significant but generally lower inhibition than that observed by the anti-MIR MoAbs (Fig. 1). The average values of inhibition obtained by MoAbs 66 and 73, with either idiopathic MG or pen-MG sera were similar (Fig. 2). Antibodies to region-64, in sera from either disease, were rare (Figs. 1, 2). MoAbs to the cytoplasmic side (155 and 124) did not exhibit significant protection against binding to the AChR of the serum antibodies from pen-MG and idiopathic MG patients (Fig. 1, top).

Anti-AChR Specificities in Serial Sera of Individual Patients

Figure 3 shows the pattern of antibodies collected from individual pen-MG patients over a period of several months, immediately after D-penicillamine cessation. Despite differences in antibody titre, the proportion of antibodies recognizing a specific region were remarkably constant. An exception was exhibited by patient no. 8 where serum antibodies to regions 64 and 66 were unstable. Excluding this patient, the standard deviations (s.d.) of the average inhibition values at varying time intervals, obtained from individual pen-MG patients' sera, were similar to the corresponding values obtained from individual idiopathic MG patients (Table 3). Both these groups had an s.d. several times lower than the s.d. obtained for sera from different pen-MG or idiopathic MG patients.

DISCUSSION

Pen-MG can provide an excellent model for understanding the pathogenesis of idiopathic MG. Study of the antibody specificities in both disorders is of great importance in the search for similarities and differences between them. In this study we present evidence that the fine antigenic specificities, of the serum

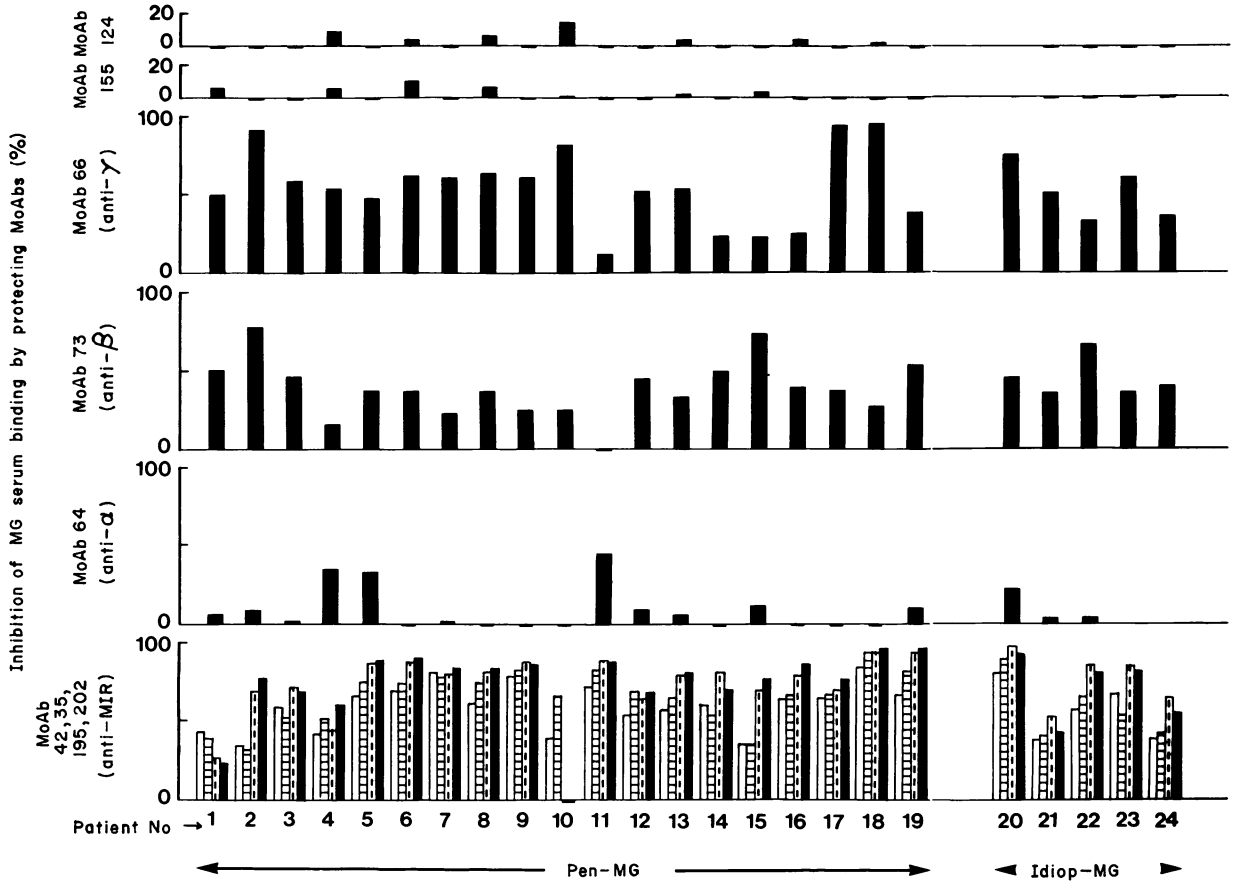

Fig. 1. Anti-AChR specificities in sera from 19 pen-MG and five idiopathic MG patients defined using nine anti-AChR MoAbs. Each bar represents % binding inhibition of the serum antibodies by the MoAbs. The effect of the four anti-MIR MoAbs is expressed by the four bars joined in the corresponding sequence of MoAb 42 (□), 35 (▤), 195 (⁝), 202 (■). Each serum was usually tested 3–4 times. s.d. was generally < 10% (usually < 5%) both within an experiment and between independent experiments.

anti-AChR antibodies, are similar in pen-MG and in idiopathic MG patients.

The competition technique used, is free of artifacts (Tzartos *et al.*, 1982) and it permits the analysis of antibodies to the intact AChR according to the location of their binding sites. It presents a major advantage in that it maps the majority of the anti-AChR human antibodies. In contrast, direct analysis of the serum antibodies by the use of peptides allows the mapping of only a small fraction of the serum antibodies. An inherent disadvantage of the competition technique is that antibodies which bind near the α-bungarotoxin binding site cannot be studied. However, this is not critical as such antibodies usually form a minority and do not seem to play a critical role in MG (Lindstrom, 1985).

The MIR was first detected by MoAbs and sera obtained from rats immunized with intact AChR (Tzartos & Lindstrom, 1980) and was subsequently found to be equally predominant in the sera of idiopathic MG patients (Tzartos *et al.*, 1982; 1985). Anti-MIR MoAbs are capable of causing experimental MG in rats, and about two-thirds of the AChR loss in mouse muscle cell lines caused by the MG sera is due to the anti-MIR antibodies (Tzartos *et al.*, 1985; 1987). In this study it was also shown that the MIR is the predominant region in pen-MG patient sera.

The observed sum of inhibition values for the antibodies of different specificities (i.e. the sum of the inhibition values obtained with the MoAbs 35, 64, 73, 66, 155 and 124) in each patient's serum was higher than 100%, with an average of 178%. This is probably due to the partial overlapping of MoAbs 73 and 66 with anti-MIR MoAbs. Nevertheless there is a general inverse relationship between the various antibody specificities of the human sera. Serum antibodies to region-64 of the AChR were rare in both pen-MG and idiopathic MG patients (Figs. 1, 2). This region also seems to be on the extracellular surface (Kordossi & Tzartos, unpublished). Detection of antibodies to the cytoplasmic surface was doubtful for both diseases since anti-cytoplasmic surface MoAbs 155 and 124 did not efficiently protect the AChR (Figs. 1, 2).

The consistent antibody specificities of individual pen-MG patients (apart from a single exception) during the few months following D-penicillamine withdrawal (Fig. 3 and Table 3), seems to agree with the constant antibody pattern which we observed earlier in sera from eight idiopathic MG patients (Tzartos *et al.*, 1982). Whiting, Vincent & Newson-Davis (1986) performed similar studies on sera from three idiopathic MG patients using a panel of mouse anti-human AChR MoAbs. They found that in two of the three patients some antibody specificities remain constant while others change over a period of time. Interestingly, the most constant antibody specificities were probably directed against the MIR. The conclusion that

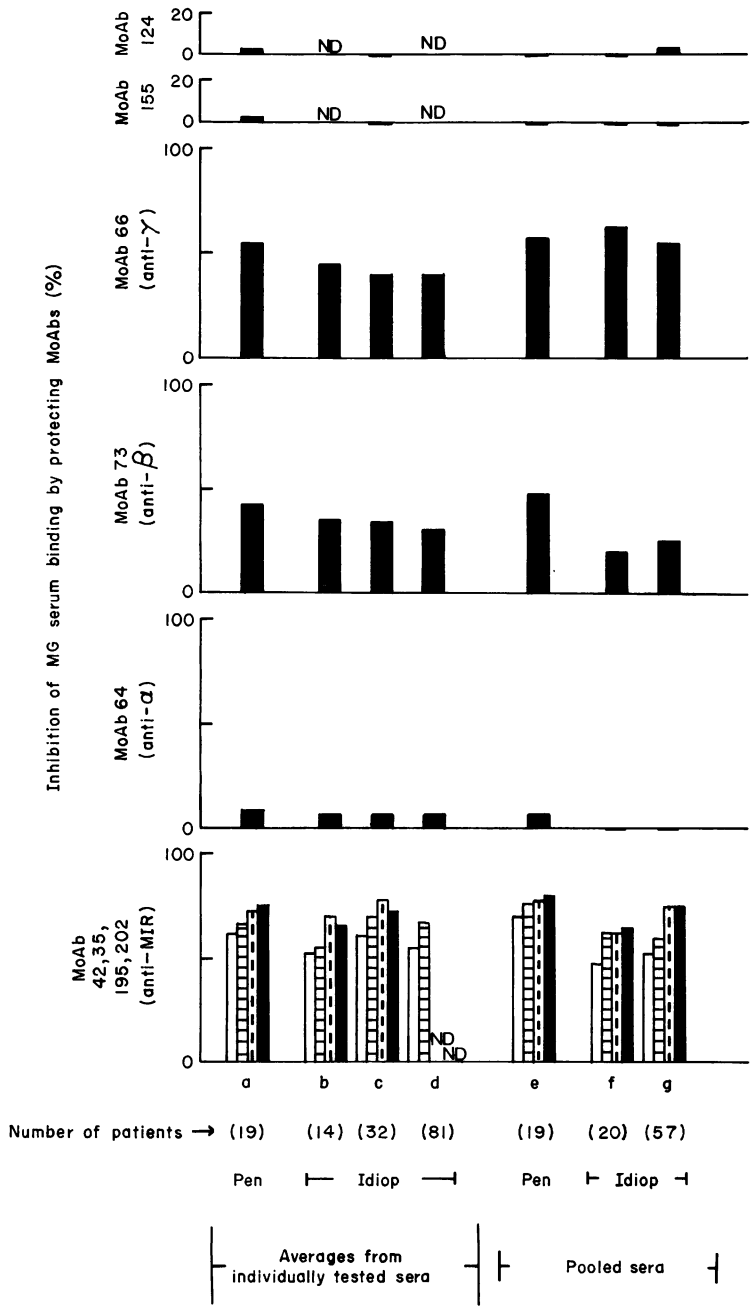

Fig. 2. Comparison of the average values of the antigenic specificities between pen-MG and idiopathic MG anti-AChR antibodies. a, values from the pen-MG sera of Fig. 1; b, c and d, values from individually tested sera from idiopathic MG patients from France (Tzartoz & Morel, unpublished), Greece and USA (Tzartos *et al.*, 1982), respectively. Data from Greek patients are from the five idiopathic MG sera of Fig. 1 for most MoAbs, or together with 27 MG sera from Tzartos *et al.* (1985) for MoAbs 35 and 73; e, sera from the 19 pen-MG patients pooled and tested as a single serum with the MoAbs; f and g, idiopathic MG sera from France (20 sera) and Greece (57 sera), respectively, pooled per population group and tested. ND, not determined.

can be drawn from the limited data of the three studies is that, in both pen-MG and idiopathic MG, the antibody specificities in sera collected from the same individual are much more constant than those among sera from different patients.

The present results provide some suggestions concerning the induction of pen-MG and idiopathic MG. The significant similarities among sera from both diseases as well as those of rats immunized with AChR (Tzartos *et al.*, 1981; 1982) strongly suggest that the immunogen, in both human diseases, is the AChR itself rather than other cross-reactive antigens. If D-penicillamine exerts its effect by modifying the AChR (Bever *et al.*, 1982), this modification is probably very similar to a putative modification of the AChR in idiopathic MG. Whether the effect of the penicillamine is at the level of the AChR or at the level of

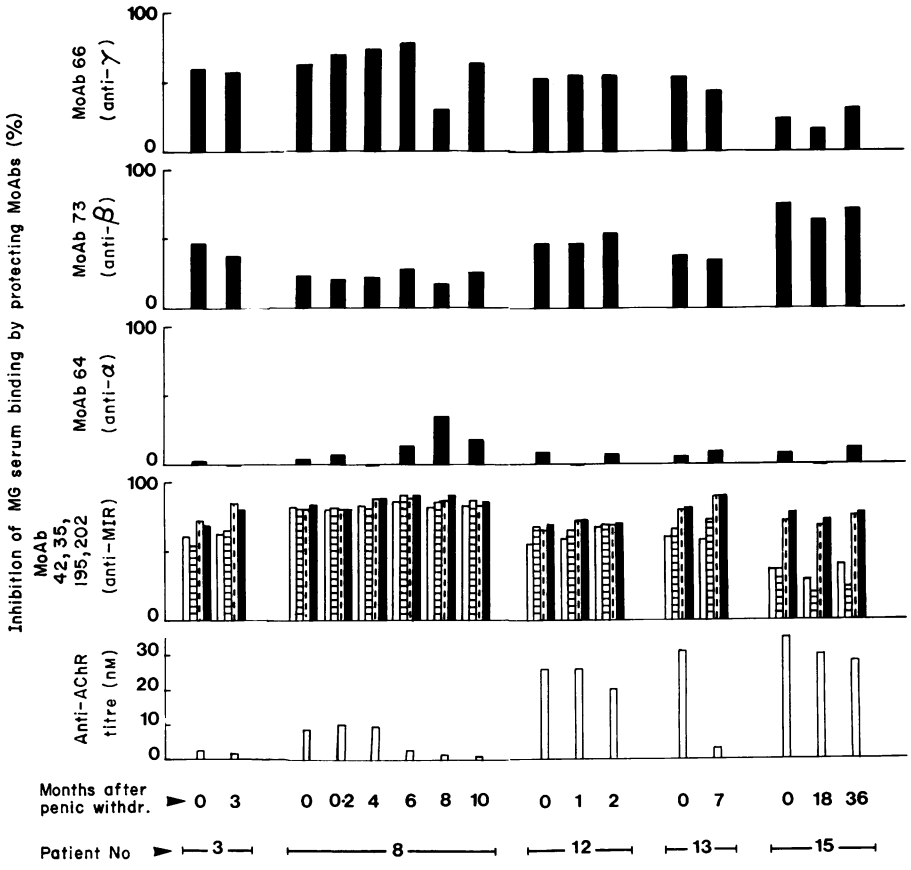

Fig. 3. Anti-AChR specificities in sera from five pen-MG patients collected at different time intervals.

Table 3. Values of standard deviation for MoAb-protection of AChR against sera from individual patients at different time intervals (longitudinal studies) or different patients with pen-MG and idiopathic MG.

	Pen-MG							Idiopathic MG	
		Within individual patients*							
Protecting MoAb	Among 19 patients*	No. 8 (6)	No. 3 (2)	No. 12 (3)	No. 13 (2)	No. 15 (3)	Mean	Among 124 patients†	Within individual patients‡
MoAb 42	15·0	1·4	1·5	5·0	1·5	4·5	2·7	24·4	5·0
MoAb 35	16·6	3·6	5·5	2·1	3·0	5·9	3·9	23·6	3·0
MoAb 195	23·4	3·1	6·0	2·8	5·0	3·7	3·8	19·0	ND
MoAb 202	23·8	2·7	5·5	1·7	4·5	3·1	3·2	23·5	ND
Average s.d. for anti-MIR antibodies:	19·7	2·7	4·6	2·9	3·5	4·3	3·4	22·3	4·0
MoAb 64	13·1	11·6	1·0	4·0	1·5	4·9	6·3	12·4	2·3
MoAb 73	19·2	3·5	5·0	4·0	2·0	4·5	3·8	22·4	2·9
MoAb 66	22·6	15·7	1·0	0·9	5·5	6·5	8·1	27·1	3·9

* Estimated from Figs. 1 and 3.
Numbers in parentheses denote the numbers of tested sera in each patient.

† Estimated from Tzartos *et al.* (1982; 1985), Fig. 1 (the five idiopathic MG sera) and Fig. 2 (the 14 French MG sera). For MoAbs 195 and 202 only 19 sera were used.

‡ Estimated from longitudinal studies of each of eight MG patients (2–10 sera per patient giving a total of 32 serum samples) of Tzartos *et al.* (1982). The s.d.s were determined separately for each patient and then averaged.

immunoregulation is not clear at the present time. The occurrence of multiple autoantibodies in some patients treated with D-penicillamine (Camus *et al.*, 1981; Morel *et al.*, 1987) possibly favours the latter hypothesis.

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