Clinical implementation of anti-acetylcholine receptor antibodies

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Abstract

A multivariate analysis of anti-acetyl-choline receptor (AChR) antibodies and clinical parameters other than treatment (modified Osserman groups, age, type of onset, sex, and thymus pathology) was performed for all incident (n = 366) myasthenia gravis (MG) cases in its white population in Denmark during the past 15 years. Sera from 244 healthy individuals and from 295 patients with diseases other than MG were analysed as controls. Formal statistics for the anti-AChR antibodies assay (immunoprecipitation RIA using crude human AChR extract) were calculated. The distribution of antibodies titres greater than 0·1 nMole/l was found to be approximately lognormal. For MG patients the 95% reference interval was 0·2–1549 nMoles/l, and in control sera the range was 0·0–0·4 nMole/l. Using 0·5 nMole/l as the cut-off level and regarding all results less than this value as normal titres, it appeared that the assay was highly specific (>99·99%) for MG. In a population of MG patients significance should be attributed to values in the range 0·3–0·4 nMole/l. The overall diagnostic sensitivity was found to be 88%. The sensitivity appeared to be proportionate to clinical severity of MG. The percentage with a normal titre was higher (16%) for early onset of MG, compared with 7% for late onset. No significant difference in relation to the frequency of "negative titre" was found in relation to sex. Anti-AChR antibodies titre was found to correlate with clinical severity, female or male gender, and pathology of thymus. The groups of MG patients were not matched for the various clinical parameters but multiple regression analysis controlling for these variables revealed independent effects of clinical severity and sex though not of age. Normal thymus (including involuted gland) and thymoma were correlated with low to intermediate titres, and hyperplastic thymus with high level of antibodies. The clinical implementation of anti-AChR antibodies is reviewed from 1976 and up to the present. The problems with false positive results are thoroughly expounded.

(*J Neurol Neurosurg Psychiatry* 1993;56:496–504)

The first assays for anti-acetylcholine receptor (AChR) antibodies detected inhibition of alpha-Bungarotoxin (BuTx) binding to AChR[1] or complement fixation,[2] but the sensitivity of those assays was less than 80%. Immunoprecipitation radioimmunoassays (RIA)[3-5] have become the standard procedure but do not distinguish between antibodies titre and functional activity of the antibodies.[6] If detergent-solubilised human AChR is used as antigen, anti-AChR antibodies are found in 75-93% of sera from patients with myasthenia gravis (MG).[4 7-9] Enzyme-linked immunosorbent assay (ELISA) has only found more limited use for anti-AChR antibodies measurement.

The correlation between anti-AChR antibodies titre and clinical parameters appears to be rather complex. Most studies find that the overall correlation between severity of MG and titre (single measurements in each patient) is weak. Ocular MG is associated with low titre and more antibodies negative cases than in generalised types of MG.[4 8 10] The occurrence of thymoma has been correlated with high titre,[4] low titre,[11] or no influence on titre.[12] The level of titre does not associate with the presence or absence of thymic germinal centres.[13] Age at onset of disease has been correlated with titre in some series,[8 14] but not others.[4] The concentration of antibodies did not correlate with sex in some studies,[4 8] but was significantly lower in males than in females in one study.[5]

Explanations of these discrepancies include technical and immunological differences between the various antibodies assays; selection bias; time criteria for serum sample varying widely in relation to onset of MG and different modalities of treatment; clinical assessment not being performed at well-defined points; inclusion of non-operated cases when comparing thymus pathology and titre; significant intercorrelation between independent variables; and inappropriate statistical methods.

This study aimed at re-evaluating the clinical implementation of anti-AChR antibodies. Only the diagnostic and prognostic applications and the distribution of antibodies in relation to clinical parameters other than treatment were considered. A prospective multivariate study of the whole incident MG patient population in Denmark during the last 15 years was carried out. Strict criteria for all variables were used, and the statistical methods were chosen with emphasis on the calculation of the effect of each variable after

adjustment for the other independent parameters.

Material and methods
Selection of blood samples
The laboratory is the only facility in Denmark receiving sera for anti-AChR antibodies analysis. The material was limited to all blood samples referred for this assay as a diagnostic test from 1 January 1980 to 31 December 1989 inclusive. During the first part of the decade many samples were referred for immunological confirmation of an already established MG diagnosis. These sera were mainly from the incident cases in the period from 1975–79 inclusive. If more than one serum sample was collected from a patient, only the first one was selected. There were no patients classified as antibodies negative cases according to the first analysis with antibodies positive samples at a later time. The control group consisted of 150 age and sex-matched healthy blood donors.

Registration of data for patients
All patients were diagnosed according to the records of the referring medical facilities. Only the most relevant diagnosis at the time of the blood sample collection was recorded for all non-MG patients. All MG patients had typical history and clinical features including response to intravenous edrophonium; most of them also exhibited electrophysiological evidence of the disease.

For all MG patients the following items were recorded: day, month and year of birth; sex; race (white or non-white groups); early or late onset of disease (that is, before or after the age of 50 years): most studies assume an arbitrary lower age limit, though epidemiological data suggest 50 years to be a more relevant cut-off;[15] date of blood sample collection; occurrence of concomitant immunosuppressive treatment or plasma exchange at time of blood sample.

The group of MG patients was divided into two subgroups according to domicile, eastern Denmark or western Denmark (East-DK and West-DK) at the onset of the disease. East-DK was defined as the part of the country East of the Great Belt (including the major islands Zealand, Lolland, Falster, Moen, and Bornholm), and West-DK as the other parts of the country excluding Greenland.

For the East-DK MG patients supplementary features were recorded: month and year for onset of MG; modified Osserman group[15] at time of sample and at maximum severity of disease (clinical assessment was performed in all cases by the author); month and year for the assignment of these groups; date for thymectomy and result of microscopic examination of thymus.

Antibody analysis
Anti-AChR antibodies were measured using an immunoprecipitation RIA with detergent-solubilised human AChR,[5] a modification of Lindstrom's assay.[4] Large batches of AChR were prepared from amputated ischaemic human leg muscles, and stored at −80°C in 5 ml aliquots to ensure the same lot of antigen over a long period of time. In general the AChR concentration was approximately 450–550 pMoles/l (expressed as alpha-BuTx binding sites). The ratio denervated AChR/normal AChR was approximately 4 to 1 (see discussion of sensitivity and false negative result). About 30 fMoles of this crude AChR extract labelled with an excess amount (approximately 20 fold, that is, 100% saturation) of ^{125}I-alpha-BuTx were used in each test tube. Control sera from healthy persons were assayed in parallel to subtract the counts per minute (CPM) corresponding to nonspecific precipitation. All results were expressed as nMoles of alpha-BuTx binding sites precipitated per litre of serum.

Before examination all samples were stored at −20°C. The current results of the various assays during the decade were used. All sera were carefully titrated to assure linear relationship between CPM and volume of sample. Normal human IgG (that is, pooled serum from healthy subjects) was added as carrier to all tubes with less than 1 μl of test serum, and each result was calculated as the mean value of 2–3 linear measurements. Using the same standard positive control serum for many years, we have found the variation of the results between the single batches to be 10–20%, and accordingly all results used in this study were calibrated using this standard positive serum as reference. In the laboratory we have found virtually no loss in antibodies activity over time for samples stored in our bank.

Statistical methods and calculations
The lowest possible measurable level was 0·1 nMole/l. The two values 0·0 and 0·1 varied at random when sera showing these results were repeatedly analysed. Accordingly, it was not possible to distinguish between the case of true zeroes and that of "zeroes" which were really censored non-zero values. The frequencies of anti-AChR titres appear to be a delta distribution (fig 1) in which a proportion of the observations may be zeros and the non-zero values follow a lognormal distribution.[16] Best unbiased estimators of mean and variance were calculated according to the method of Aitchison.[16] Alternatively, antibodies titres were transformed using the base 10 logarithm, and zero values were set to 0·1 enabling them to be included in the transformation. Curve fitting (with lognormal curves) was performed using the method of the least sums of squares for the residuals.

For cross-tabulations the Chi-Square test of independence was used. Hypotheses about population logarithmic means were estimated by the independent samples *t* test. The Mann-Whitney test was always used in parallel in view of the possibility of more than moderate violation of the assumptions for the *t* test. The results of the parametric and nonparametric tests were in agreement, see tables
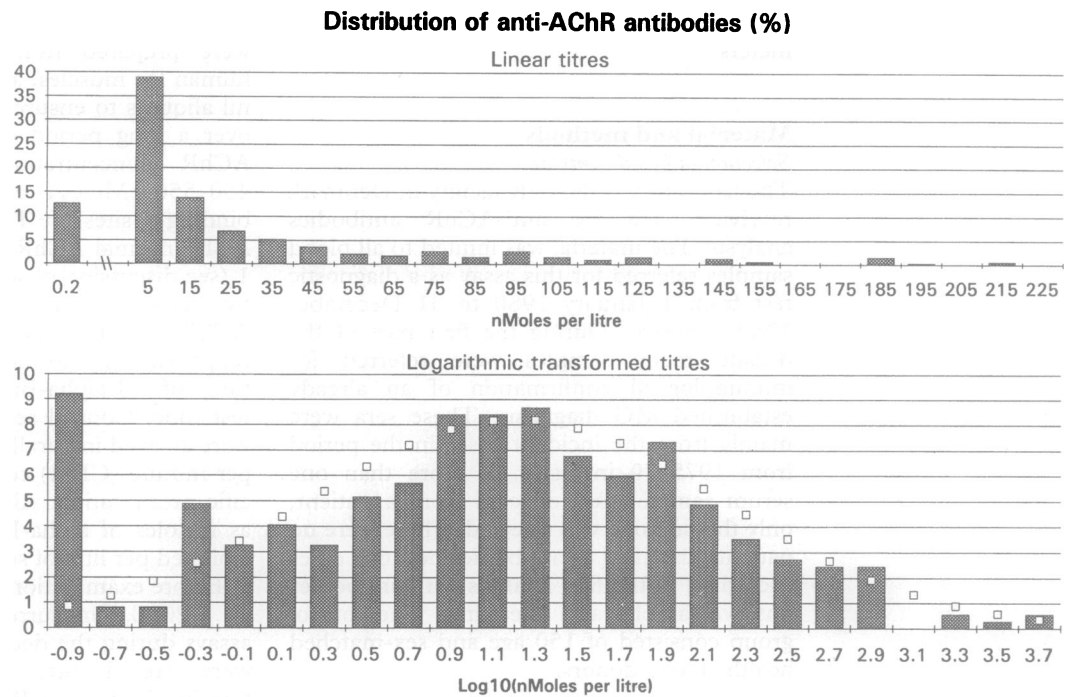
Figure 1 Distribution (%) of anti-AChR antibodies titres. Categories axis: midpoint values for the sampling intervals. Upper panel: Linear titres. Sampling increment was 10 nMoles/l. Intervals: <0·5, 0·5–9·9, 10–19, .. 120–129. The category axis was truncated at the latter interval. Lower panel: Logarithmic transformed titres. Sampling increment was 0·2. A fitted lognormal curve has been superimposed.

6 and 7. Multiple regression analysis was applied with the aim of controlling for the different apportionments in relation to the various clinical variables.

Results

Nine hundred and twelve samples were eligible, 539 non-MG sera and 373 MG samples. Three non-white MG sera were excluded. Four MG cases were excluded from the study as no serum samples taken before immunosuppressive treatment were available. Of the remaining 366 MG samples, 45 sera (all non-thymoma cases) were taken after thymectomy (12%), but this was not considered to be a disqualifying feature. The geographic distribution of the MG samples was 199 sera from East-DK and 167 sera from West-DK. The East-DK samples were collected from all known incident cases from 1975–89 within the geographical boundaries.[15] The West-DK sera represented more than 90% of the expected number of incident cases from that area during the same period of time. Accordingly the use of this material is a reasonably accurate approach to an unbiased analysis of the whole patient population within the country during a well-defined length of time.

Table 1 summarises the results from all 539 non-MG sera. Most non-MG cases were 0·0 nMole/l negative, but the range was 0·0–0·4 nMole/l. Previously 40 other sera from patients with autoimmune and neuromuscular diseases other than MG were analysed by the same method,[5] the range for

those samples also being 0·0–0·4 nMole/l. To minimise the risk of false positive results we have, during the past decade, set "positive

Table 1 Anti-AChR antibodies analysis of 539 nonmyasthenic sera.

	n	nMoles/l
Healthy individuals: total	244	0·0–0·3
Age and sex-matched blood donors	150	0·0–0·3
Referred persons	78	0·0–0·3
Monozygotic twin to MG patient	1	0·0
Relatives of MG patients	15	0·0–0·2
Neurology: total	205	0·0–0·4
Amyotrophic lateral sclerosis	26	0·0–0·2
Dystrophia myotonica	6	0·0–0·4
Eaton-Lambert syndrome	7	0·0–0·2
Multiple sclerosis	7	0·0–0·4
Muscular dystrophies	11	0·0–0·3
Myopathy (congenital)	3	0·0–0·2
Myopathy (mitochondria)	4	0·0–0·4
Myopathies of later onset	18	0·0–0·3
Polymyositis	8	0·0–0·2
Polyneuropathy	17	0·0–0·1
Polyradiculitis	5	0·0–0·2
Spinal muscular atrophies	2	0·0–0·1
Other diseases	91	0·0–0·4
Rheumatology: total	25	0·1–0·4
Myalgia of unknown origin	6	0·0–0·2
Polymyalgia rheumatica	4	0·0–0·2
Rheumatoid arthritis	7	0·0–0·2
Systemic lupus erythematosus	2	0·3–0·4
Temporal arteritis	2	0·1–0·2
Other diseases	4	0·0–0·1
Psychiatry (neuroleptics therapy)	29	0·0–0·3
Other disorders: total	36	0·0–0·2
Acquired immunodeficiency syndrome	2	0·0–0·1
Cholinesterase deficiency	1	0·0
Diabetes mellitus	7	0·0–0·2
Lymphoma (non-Hodgkin)	3	0·0–0·2
Myxedema	5	0·0–0·1
Neoplasms (non-lymphoma)	3	0·0–0·1
Toxic diffuse goiter	4	0·0–0·2
Other diseases	11	0·0–0·2

Table 2   Clinical features

All Danish MG patients n = 366

Type of onset

Early <50 y	52%
Late>=50y	48%

Sex

Females	62%
Males	38%

Antibody negative[1] 12%

Female/male ratios

All	1·6
Early onset	2·8
Late onset	0·9

Early/late onset ratios

All	1·1
Females	1·7
Males	0·6

East Danish MG patients

Modified Osserman groups (%)

	females n = 123		males n = 76		all n = 199	
	A	B	A	B	A	B
1	12	6	26	13	18	9
2A	27	16	24	17	26	17
2B	39	42	22	30	33	38
3	7	8	16	20	10	13
4	15	28	12	20	14	24

[1]defined as titre <0·5 nMole/l
A: at time of sample collection
B: at time of maximum severity of disease

titre" to greater than or equal to 0·5 nMole/l, and regarded 0·3–0·4 nMole/l as equivocal values. As a safety precaution all low titre sera (0·3–2·0) have always been assayed twice. More than 3500 samples have been analysed in our laboratory during the decade, and no false positive results (defined as > = 0·5 nMole/l) have been found.

Table 2 outlines the clinical features for the MG patients. The incidences of the various variables for the East-DK and the West-DK groups were similar, and consequently the East-DK subpopulation of patients was representative of the whole MG population. The accumulative probability for the time interval from onset of MG to the time of the blood sample was calculated on the basis of the 199 East-DK patients. The median was 3·0 years, and many of the patients with a shorter interval were of categories modified Osserman groups 3 and 4. Table 2 also demonstrates deterioration from modified Osserman groups 1 + 2A towards higher categories after the time of the sample collection. Accordingly the distribution of antibodies titres primarily reflects the late initial progressive phase of the disease not subjected to immunosuppressive treatment. The apportionment of modified

Osserman groups at maximum severity of MG differed statistically (Chi-Square p = 0·025) with more male cases than expected in group 1 and 3.

Figure 1 displays the frequencies of antibodies titres measured on a linear interval scale with a sampling increment of 10 nMoles/l. The mode for the histogram was located at and below the cut-off point, and the graph was not symmetrical. The median for the 366 patients was 12 nMoles/l, and the arithmetic mean value was 106 nMoles/l, see also table 3. Figure 1 also shows the frequencies of the logarithmic transformed antibodies titres with a sampling increment of 0·2 and a superimposed normal curve. The distribution of logarithmic titres greater than linear values 0·1 nMole/l was approximately lognormal. Moreover, the distributions of the transformed antibodies titres for females, males, early onset, and late onset were approximately lognormal (not shown in any figures).

In table 3 different statistics have been calculated for the interval-level data as well as for the logarithmic transformed and fitted data. The lower 95% reference limit was calculated as 0·1–0·2 nMole/l. The graph (fig 1) clearly demonstrates the problem with false negative cases. At the cut-off point, log(0·4 nMole/l) = – 0·39, there was even a slight increase in frequency, and a significant part of the left tail has been excluded. The 0·0–0·1 nMole/l cases make a total of more than 9%.

Specificity and sensitivity of the antibodies analysis can thus be calculated (table 4). In spite of the finding of 20% "negative" titres in ocular MG compared with 8% in severe cases and of 29% normal titres in early onset ocular MG compared with 12% in late onset ocular MG, all the Chi-Square p values in relation to clinical severity were not significant. A statistical type 2 error may have been operative, since the number of cases with normal titre was small in all groups. Consequently, from a diagnostic point of view sensitivity may be regarded as proportionate to clinical severity. Antibodies negative cases were more frequent for early onset of MG (16%) than for late onset (7%) (p = 0·01). On the other hand, sex and anti-

Table 4   Diagnostic specificity (%) and sensitivity (%) for the anti-AChR antibodies assay (RIA)

Specificity* >99·99

Sensitivity† 88

Sex		Onset of MG	
Females	Males	Early	Late
90	86	84	93

Clinical severity of MG Osserman groups	Onset of MG		All
	Early	Late	
Ocular (1)	71	88	80
Mild generalised (2A)	86	88	86
Severe generalised (2B–4)	89	98	92

The table has been calculated for the 199 East Danish cases.
*No false positive (FP) cases were found, but as a safety precaution FP was set to: <1/10000.
†Cut-off level for normal antibodies titre was set to <0·5 nMole/l.

Table 3   Statistics for anti-AChR antibodies in all 366 MG cases

	Linear values		Logarithmic transformed values	
	Arithmetic	Aitchison	Arithmetic	Lognormal fitted
Mean	106	124	1·007	1·211
SEM	21	56	0·055	0·057
SD	395	1063	1·056	0·989
Median	12			
Mode	0·1			
95% reference interval*			0·1–1315	0·2–1549

Unity for all values: nMoles per litre; Aitchison: Aitchison's estimators; SD: standard deviation; SEM: standard error of mean.
*antilog (mean + − 2*SD), that is, linear values.

Table 5   Mean log anti-AChR antibodies titre in relation to modified Osserman groups at the time of sample collection

Students t test group	n	titre	p values: group versus group				
		log(nMoles/l)	1	2A	2B	3	1 + 2A
1	35	0·3778					
2A	51	0·9042	0·02				
2B	65	1·1467	0·00	NS			
3	20	1·2222	0·01	NS	NS		
4	28	1·4979	0·00	0·02	NS	NS	
1+2A	86	0·6899					
2B + 3 + 4	113	1·2474					0·00

The table has been calculated for the 199 East Danish cases. NS: not significant. Non-parametric test (Mann-Whitney) confirmed the significant values.

Table 6   Mean log anti-AChR antibodies titre in relation to sex, type of onset, and thymus pathology

Students t test	n	titre	p values: group versus group			
		log(nMoles/l)	Males	Late	Normal	Thymoma
Sex						
Females	123	1·2384	0·00			
Males	76	0·6307				
Onset						
Early	126	1·0890		NS		
Late	73	0·8636				
Thymus histology						
Normal	18	0·7824				
Thymoma†	14	1·0478			ns 0·03	
Hyperplasia	57	1·4974				0·04

The table has been calculated for the 199 East Danish cases.
†All serum samples collected before thymectomy.
NS: not significant. Non-parametric test (Mann-Whitney) confirmed the significant values.

bodies titre as a dichotomy, normal or abnormal, appeared to be independent, though the absolute value of antibodies titres was certainly correlated to sex, see below.

The correlation between mean logarithmic antibodies titre and modified Osserman groups can be summarised as in table 5. There was no significant difference between the localised and mild groups: 1 and 2A, nor were there significant differences between the more severe cases: groups 2B, 3, and 4. Conversely, group 1 differed significantly from all other groups than 2A, and group 2A had lower mean titre than that of group 4. Figure 2 shows the increasing level of antibodies versus Osserman groups. All the significant differences also held true for female gender but not for males, a finding which

may be attributable to the lower level of antibodies for males, see below.

The mean antibody level was significantly lower in sera from male patients compared with that of sera from female patients (table 6). There was no significant difference in relation to type of onset of MG. Hyperplasia of the thymus was associated with higher antibodies concentration compared with normal thymus or thymoma (table 6). There was no significant difference between the antibody titres of patients with normal thymus and thymoma. Antibody negative cases with thymoma were not observed. Figure 2 summarises the correlation between anti-AChR antibodies titre and the clinical variables.

To estimate the possible effect of confounders, multiple regression analyses were performed. Mathematical models controlling for the different variables revealed independent effects of clinical severity and of female or male gender (table 7). Additional calculations not including the transformed zero values showed independent effect of the same variables, proving that the log 0·1 values did not have any undue influence on the analysis. When each of the factors is used alone (table 7, A), the most importance must be assigned to Osserman groups (standardised regression coefficient 0·28), although sex appeared to be of similar influence (−0·25). Since antibody (the dependent variable) is in log units, the coefficients can be interpreted approximately in percentage terms. Thus after statistical adjustment for the other independent variables, male antibodies titre can be estimated to be about 25% less than in females. Looking at the clinical assessment as a dichotomy, mild or severe (table 7, B), the antibodies concentration in severe MG can be estimated to be about 23% higher than in the mild cases. On the other hand, the residuals were still significant when these clinical variables were accounted for, and consequently additional factors of importance for the homeostasis and activity of the antibodies must be operative.

Figure 2   Correlation between logarithmic anti-AChR titre and clinical parameters. Logarithmic mean values are indicated by cubes.

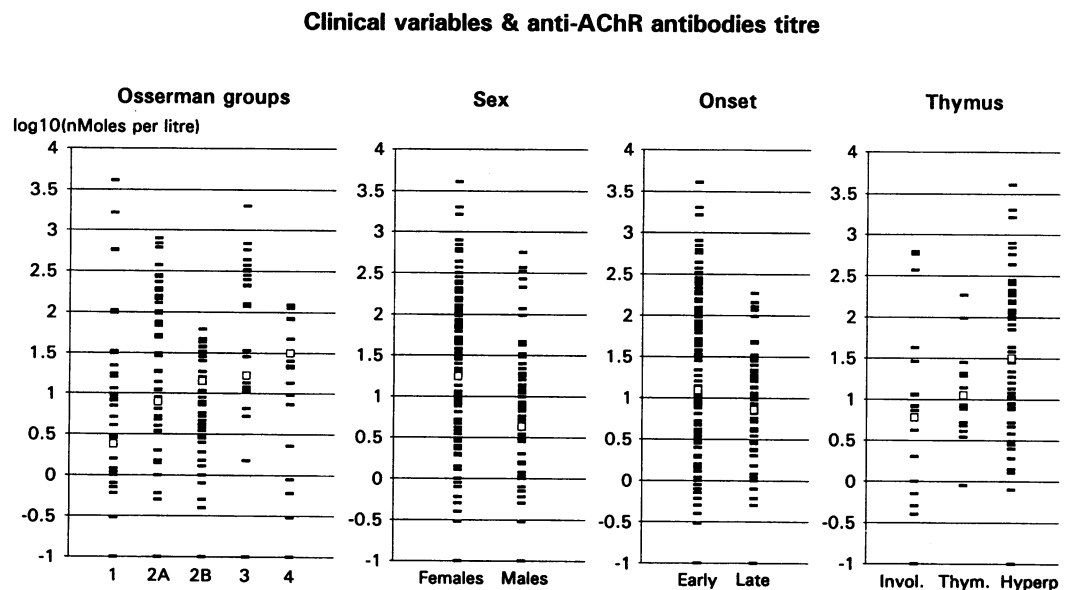
Clinical variables & anti-AChR antibodies titre

Table 7 Significant mathematical models for mean log antibody titre. Regression analysis with the objective of assigning relative importance to each of the independent variables rather than predicting antibody titres.

A
$\log(abs_{conc}) =$ 0·55 (+ −0·18)
            +0·24 (+ −0·06) *Oss$_{sample}$
            −0·55 (+ −0·14) *Sex
B
$\log(abs_{conc}) =$ 0·93 (+ −0·13)
            −0·55 (+ −0·15) *Sex
            +0·50 (+ −0·14) *Mild/Severe$_{sample}$

Standardized regression coefficients

	A	B
Sex:	−0·25	−0·25
Oss$_{sample}$:	0·28	
Mild/Severe$_{sample}$:		0·23

Values in brackets (+ −standard error). P values for all constants: <0·001. Age parameters, such as age at sample collection or type of onset, did not show independent linear correlation. Abs: antibodies; Oss: modified Osserman group; Mild: groups 1 + 2A; Severe: groups 2B + 3 + 4; Sample: at the time of sample collection.
Coding of variables: Oss groups 1 = 1; 2A = 2; 2B = 3; 3 = 4; 4 = 5 Sex Females = 0; males = 1 Mild/severe Mild = 0; severe = 1 Onset of MG Early = 0; late = 1.

Discussion

Distribution of anti-AChR antibodies

The distribution of the frequencies of titres expressed as interval-level measurements (fig 1) with mode located below the cut-off point, median at 12 nMoles/l, and upper range greater than 3000 nMoles/l, is an important clue to the understanding of the conflicting results of correlation analysis of anti-AChR antibodies titre versus various clinical parameters, as reviewed in the introduction. Scatterplots of interval-level titres versus any of the clinical variables showed a weak linear trend, but due to the disseminated location of the points any linear fit was problematic (not shown in any figures). On the other hand, logarithmic transformation (fig 1) resulted in an approximately lognormal distribution of the frequencies of titres. This agrees with Kornfeld et al.[13] This group used rat AChR as antigen in their assay, yielding only 55% positive MG sera. Furthermore, their patient population was weighted towards higher age groups. The lognormal distribution of the anti-AChR antibodies is thus confirmed for human AChR and for a whole patient population within a country.

In table 1 the results of routine examination of 539 non-MG sera for anti-AChR antibodies are listed. A total of 150 samples were relevant unbiased controls, 473 sera came from patients with MG as a possible differential diagnosis, and 16 sera were from first-degree relatives of MG patients, including 1 monozygotic twin sister. The high prevalence of anti-AChR antibodies (low titres) in relatives, proposed by Lefvert et al[17] was not confirmed, which agrees with Vincent et al.[9] On the basis of the review of false-positive (see below) and the examination of true-negative results (table 1), the conclusion must be that the assay for anti-AChR antibodies is highly specific as a diagnostic test (table 4). This agrees with many other studies.[4 5 8 9 13 18 19] If a conservative cut-off point is applied (that is, positive > = 0·5 nMole/l), false positive outcome is extremely rare. More significance

should be attributed to values in the range 0·3–0·4 nMole/l in a selected population of patients in whom MG is estimated as probable and for whom relevant differential diagnoses are unlikely.

Sensitivity of the assay and false negative result

The sensitivity of the analysis appeared to be proportionate to the clinical severity of the disease (table 4). A negative result of the antibodies assay does not exclude MG. Antibodies negative MG cases were found to be more common for early onset of the disease than for late onset, though sex did not appear to affect the frequency of antibodies negative cases (table 4). A few sera are positive only if the antigen is intrajunctional (normal) AChR, the antigen source is extraocular muscle, or the AChR extract is not saturated with BuTx.[9 20] Anti-AChR antibodies which bind to the BuTx site of the receptor can be demonstrated in more than 30% of MG sera,[1] but these are the only anti-AChR antibodies demonstrable in just a few per cent of MG sera.[18] The RIA can be modified to detect these antibodies reliably.[18] Other modified protocols further reduce the proportion of antibodies negative cases.[9] The use of heterologous AChR reduces positivity; sensitivity with rat AChR is 55%,[13] fetal calf AChR 74%,[14] and with monkey AChR 29%-79%.[21]

False positive result

Biological false-positive results appear to be rather infrequent. Low titre positive outcome has been reported in non-MG patients: amyotrophic lateral sclerosis;[22-24] penicillamine-treated patients without MG;[8 25] thymoma without MG;[9 26] neonates to anti-AChR antibodies positive MG mothers;[27] bone marrow transplantation;[28] elderly and Down's syndrome Japanese but not white subjects;[29 30] tardive dyskinesia;[31] patients with high antithyroid autoantibodies (borderline titres);[30] primary biliary cirrhosis and patients with mitochondrial antibody.[32] The sera from many of these patients are not false-positive in a true sense, and this matter will be reviewed in the following paragraphs.

In first-degree relatives of MG patients the prevalence of anti-AChR antibodies (low titres) and anti-idiotypic-AChR antibodies was found to be 54% and 37% respectively.[17] The natural occurrence of anti-idiotypic-AChR antibodies has been disputed.[33] Furthermore, this high incidence of positivity in relatives has not been confirmed by this study or by other groups.[9] One out of three monozygotic twins (without clinical MG) to female MG patients was anti-AChR antibodies positive.[9]

False-positive immunoassay for anti-AChR antibodies has been reported for 19 of a total of 171 cases with amyotrophic lateral sclerosis (ALS).[22-24] A closer look reveals that no anti-AChR antibodies were found in 10 of these cases; the antibodies were crossreacting with BuTx, which could be attributed to experimental snake venom therapy in all cases.[22 24] Two of these patients exhibited

decremental response on electromyography and also clinical signs in accordance with MG,[23][24] so they may well be diagnosed as having MG as well as ALS. Of the remaining 7 patients, very low titre (0·1–0·4 nMole/l) was found in 5 cases and low titre (0·8 and 4·1 nMoles/l) in 2 cases. No follow up data have been published for these patients. In Denmark we have found no ALS cases with significant anti-AChR antibodies titres (table 1).

Anti-AChR antibodies in penicillamine-treated patients without MG were first reported in 1980,[25] but no follow up data are available. Since 1975 it has been known that MG could be induced by penicillamine in patients with rheumatoid arthritis.[34] The fine specificities of the anti-AChR antibodies in sera from these patients do not differ from the antibodies in sera from idiopathic MG.[35] Penicillamine probably produces MG by initiating a new autoimmune response in most of these cases rather than by enhancing ongoing autoimmunity. Usually antibody levels fall rapidly after stopping penicillamine treatment.[35] The author has diagnosed 5 penicillamine-induced MG cases in Denmark since 1980. In 4 of these cases the clinical signs of MG disappeared within 5 months after penicillamine treatment was discontinued, and serial measurements showed decrease in the anti-AChR antibodies titres during the same period of time. Ultimately the antibodies could no longer be detected. In one case the antibodies level and the clinical condition did not change during a follow up period of 4 years, proving that in some cases the association between penicillamine and MG could be due to chance or autoimmune predisposition. Further evidence for this view is the co-existence of MG, rheumatoid arthritis, or other autoimmune diseases in the absence of penicillamine-treatment.[36] Until now we have not found anti-AChR antibodies in penicillamine-treated patients without MG in Denmark.

Other case histories with chemically induced autoimmunity have been published by Lieberman et al,[31] who examined sera from 34 patients with neuroleptic-induced tardive dyskinesia. Three sera had anti-AChR antibodies titre with range 0·8-1·4, and 12 sera were in the range 0·3-0·5. It is not stated whether the patients presented MG symptoms or not. The titres were inversely correlated to the period of time off neuroleptics. At least 9 out of the 29 psychiatric cases examined in our laboratory (table 1) were on neuroleptics at the time of sample collection and the other 20 patients had discontinued such a treatment shortly before, but all 29 sera were "0·0" negative.

Anti-AChR antibodies have also been reported in a few sera from non-MG patients with thymoma.[8][9][26] A systematic examination of this topic has not been published. In larger series of operated thymomas 53%-73% were also diagnosed as MG,[37][38] and other autoimmune diseases were manifest in 7% of the patients in the series.[37] The period between the finding of thymoma and the onset of MG

may be up to 10 years.[39] Accordingly the occurrence of anti-AChR antibodies in non-MG thymoma cases may possibly anticipate later development of MG symptoms.

Anti-AChR antibodies were found in 11 out of 54 (20%) bone marrow transplanted (BMT) patients.[28] Some of these patients developed MG 1–3 years after allogeneic BMT. The titres in the non-MG patients were low (0·3-4·8 nMoles/l), and the antibodies response was transient in 4 cases. Graft-versus-host disease has been implicated, and in one of the cases with MG after BMT the autoimmune disease was probably of donor origin.[28]

Methodological details and thus the values obtained for AChR binding by normal sera differ in various laboratories. Nevertheless, great care should be taken in the assignment of "false-positive" to an anti-AChR antibodies titre >0·4 nMole/l. Garlepp et al[19] have argued for a symptomatic threshold. They followed a patient with MG and Graves' disease during 5 years. Both before MG symptoms and after remission a low anti-AChR antibodies titre was found, and during the active phase of MG the titre was approximately 3 times higher. Further evidence for the concept of a symptomatic threshold is that significant levels of anti-AChR antibodies may also be found after remission of MG.[4] Thus it is clear that the mere occurrence of anti-AChR antibodies in serum is not sufficient evidence for the diagnosis of active autoimmune MG. This is also emphasised by the fact that 16 out of 17 children to 15 MG mothers had anti-AChR antibodies at birth,[27] but only 2 infants had neonatal MG.

Correlation between anti-AChR antibodies and clinical parameters

It is evident from tables 6–8 that the correlation between anti-AChR antibodies titre and clinical parameters is rather complex. Ocular/mild MG and male sex indicated lower titre than severe generalised MG and female gender. As substantiated in the Results section, these variables do not explain all the variability in antibody titres. A major additional variable may be the heterogeneity of the anti-AChR antibodies.[7][10]

Most studies have suggested that anti-AChR antibodies titre correlates with clinical condition, and that the association was most apparent for ocular MG versus generalised cases.[4][8][10] This was confirmed by this study. In addition, titres from patients with mild generalised disease (group 2A) differed significantly (table 5) from those with more severe disease (group 4), which is in accordance with Tindall.[10]

As stated earlier, all possible combinations of antibodies level in relation to thymoma have been described. Thymoma has been correlated with high titre.[4] Other reports suggest that the occurrence of a thymoma does not in itself predict any influence on titre.[12] On the other hand, it appears from this study that thymoma cases exhibit low to median titres, provided that the samples are collected before

thymectomy (table 6), which agrees with Bartoccioni *et al.*[11] A possible explanation for the finding of high titre in thymoma cases may be the inclusion of post-operative samples, that is, exacerbation after removal of thymoma[40] could significantly bias results. The author's own experience is further evidence for this point of view, as exacerbation of MG (post-operative deterioration in muscular strength and increase in titres lasting up to 3 years) was observed in all thymoma MG cases operated on in East DK during the last decade.[41]

Lindstrom *et al*[4] found that neither presence of anti-AChR antibodies nor titre appeared to correlate with age. However, Limburg *et al*[8] and Mantegazza *et al*[14] provided evidence for an association between titre and age at onset of MG. Tables 4, 6 and 7 show that this study agrees with Liindstrom *et al*,[4] with the exception that antibodies negative cases appeared to be more frequent for early onset of MG compared with late onset (table 4).

Table 6 shows that men had significantly lower mean antibodies level than women. Lindstrom *et al*[4] were unable to demonstrate such a correlation, and Limburg *et al*[8] rejected this possibility although their p value was less than 0·02, referring to the high number of men with only ocular MG in the material. Table 2 shows that the same feature might influence the present analysis. However, the results of the multiple regression analysis (table 7) exclude the possibility that this association can be explained solely by differences in apportionment to clinical groups or in age. Gammeltoft and Somnier[5] found this correlation in a more limited study, and it is now confirmed by this study of the whole MG population in Denmark. Associations between sex and autoantibodies other than anti-AChR (anti-thyroid and antinuclear antibody) in MG patients is described by Sagar *et al.*[42]

Thus multivariate mathematical models (table 7) must be applied in the prediction of antibodies level rather than more simple bivariate models. If the models in table 7 are implemented on longitudinal change in titre in the single patients, variable sex is nullified. Accordingly the models predict a much stronger correlation between change in titre and clinical course for the single patient, and this has indeed been observed.[43] Assays measuring functional activity of the antibodies[6] may add to the correlation between change in antibodies titre and clinical status.

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