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Age- and sex-related resistance to chronic experimental autoimmune myasthenia gravis (EAMG) in Brown Norway rats

A. HOEDEMAEKERS, Y. GRAUS, P. VAN BREDA VRIESMAN & M. DE BAETS
Department of Immunology, University of Limburg, Maastricht, The Netherlands

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SUMMARY

The influence of age and sex on the induction of chronic EAMG was analysed. Aged male rats, immunized with Torpedo acetylcholine receptor (tAChR), showed no clinical signs of disease or AChR loss. Immunization of young male Brown Norway (BN) rats resulted in both clinical signs of disease and 65% AChR loss. In contrast, both young and aged female BN rats showed comparable AChR loss (58% and 50%, respectively), although aged female rats did not develop clinical signs of disease. Differences in antibody titres, isotype distribution, fine specificity or complement activation could not account for the observed resistance. These results suggest that resistance against EAMG in aged rats is due to resistance of the AChR against antibody-mediated degradation, or to mechanisms able to compensate for AChR loss.

Keywords myasthenia gravis ageing autoimmunity resistance susceptibility

INTRODUCTION

Myasthenia gravis (MG) is caused by autoantibodies against the nicotinic acetylcholine receptor (AChR). Symptoms of both MG and EAMG result from an antibody-mediated destruction of AChR at the neuromuscular junction. Several pathogenic mechanisms are held responsible for loss of functional AChR. These include antibodies that cross-link AChR causing increased internalization, called antigenic modulation [1,2]; anti-AChR antibodies also activate complement, resulting in focal lysis of the postsynaptic membrane; third, antibodies that interfere with ligand binding or ion channel function cause impairment of AChR function [3,4].

The incidence of MG in humans is both age- and sex-related. The relative incidence is highest in women in the second and third decades. The incidence in men peaks in the sixth and seventh decades [5,6].

EAMG is an animal model that closely resembles human MG. It can be induced by active immunization in a large variety of species with purified AChR or by passive transfer of polyclonal or monoclonal anti-AChR antibodies [7–9]. Susceptibility to EAMG varies between several rat strains, and is intermediate in Brown Norway (BN) and Lewis rats that are most commonly used [10]. However, aged BN rats are relatively resistant to both passive transfer and chronic EAMG [11], apparently because of a decreased antibody-mediated degradation or other mechanisms that compensate for AChR loss.

Several immunological changes have been shown to occur in senescence. Both T cell and antibody responses to foreign and autoantigens decrease [12,13], whereas the spontaneous occurrence of circulating autoantibodies increases with ageing [14]. Increased T cell autoreactivity also occurs, but is rarely accompanied by overt disease [15]. An age-associated decline in susceptibility to experimental autoimmune models of systemic lupus erythematosus (SLE), encephalomyelitis and thyroiditis is usually associated with a decreased activity of T helper cells and lower antibody titres [16,17].

In addition to epidemiological evidence in humans, experimental evidence from spontaneous and induced animal models of autoimmune disease shows that sex hormones play an immunomodulatory role [18,19].

In the present study, the influence of age and gender on induction of chronic EAMG, and possible differences in pathogenic mechanisms that cause AChR degradation, were further investigated. The results of this study might elucidate putative protective mechanisms operative in the milder clinical form of MG in older patients [5].

MATERIALS AND METHODS

Animals

Inbred male and female BN rats were bred at the local breeding colonies of the Department of Experimental Animal Services and originated from TNO Leiden, The Netherlands. All animals were

Y.G. current address: The Netherlands Cancer Institute, Division of Immunology, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

Correspondence: Dr M. de Baets, University of Limburg, Department of Immunology, PO Box 616, 6200 MD Maastricht, The Netherlands.

bred under specific pathogen-free conditions. The animals were killed by exsanguination under ether anaesthesia.

Induction of chronic EAMG

AChR from electric organs of *Torpedo californica* (tAChR; Pacific Biomarine, Venice, CA) was purified by affinity chromatography using cobra toxin coupled to Sepharose 4B (Pharmacia LKB, Woerden, The Netherlands) [20]. Rats were immunized at the base of the tail with 10 μg/100 g body weight purified tAChR emulsified in Freund's complete adjuvant (FCA; Difco Labs, Detroit, MI). Four (female rats) or 6 (male rats) weeks after primary immunization rats were boosted with 10 μg/100 g body weight tAChR in Freund's incomplete's adjuvant (FIA; Difco Labs). Control rats received an equal volume of PBS in FCA or FIA.

Clinical assessment

The severity of EAMG was scored by measuring weight loss and muscular weakness. The animals' muscle strength was assessed by their ability to grasp and lift repeatedly a 300-g rack from the table, while suspended manually by the base of the tail for 30 s. Clinical scoring was based on the presence of tremor, hunched posture, strength and fatiguability. Results were expressed as 0 (no obvious abnormalities), + (no abnormalities before testing, but reduced strength at the end), + + (clinical signs present before testing, i.e. tremor, head down, hunched posture, weak grip), + + + (severe clinical signs present before testing, no grip, moribund) [21,22].

Determination of AChR concentration

The concentration of AChR was determined in the complete carcass as described previously, with minor modifications [7,23]. Briefly, frozen tissue was homogenized and AChR was extracted with 2% Triton X-100 (Sigma, Brunschwig Chemie b.v., Amsterdam, The Netherlands). An aliquot of 250 μl of each extract was labelled with 2×10^{-9} M ^{125}I-α-bungarotoxin (α-BT), incubated overnight with excess rat anti-AChR IgG and precipitated by goat anti-rat antibodies. AChR concentration was expressed as pmoles ^{125}I-α-BT precipitated.

Determination of anti-AChR antibody titres and isotypes

The concentration of antibodies reactive with rat AChR (rAChR) from individual sera was determined by radioimmunoassay (RIA) using rAChR from denervated muscle labelled with ^{125}I-α-BT from *Bungarus multicinctus* (Sigma) as previously described [7]. Titres were expressed as nmoles α-BT precipitated per litre. Corrections for interassay variability were made based on serial dilutions of an EAMG standard control serum pool tested in each assay.

Pooled serum from young and aged rats was tested for anti-tAChR antibody isotypes by ELISA. First, anti-tAChR antibody concentrations were measured by ELISA to assess the serum dilution in which equal amounts of anti-tAChR antibodies were bound to immobilized tAChR. Polyvinyl 96-well microtitre plates were coated with 50 μl tAChR (5 μg/ml) for 1 h at 37°C. Plates were then incubated with PBS containing 0·5% bovine serum albumin and 0·5% Tween-20 (P–BSA–T) for 15 min at room temperature. After washing three times with 0·5% Tween, plates were incubated with 50 μl serum diluted in P–BSA–T for 1 h at room temperature, followed by peroxidase-conjugated polyclonal rabbit anti-rat antibodies (1·3 μg/*l*) (Dako, Glostrup, Denmark). After washing again with 0·5% Tween, the colorimetric reaction was developed by adding 100 μl of 0·1 M sodium-acetate buffer pH 5·5, containing tetramethylbenzidine (10 mg/ml) and 0·01% H_2O_2. After 10 min the reaction was stopped by adding 50 μl 4 N H_2SO_4. Optical density (OD) was measured at 450 nm (Titertek Twinreader; Amstelstad, Amsterdam, The Netherlands). To determine the isotype distribution, polyclonal rabbit anti-rat isotype antibodies were used instead (1:250–1:500 dilution) (a generous gift from Professor H. Bazin, VCL, Louvain, Belgium) for 1 h at room temperature, followed by 50 μl peroxidase-conjugated swine anti-rabbit antibodies (1·3 μg/*l*) (Dako). The colorimetric reaction was developed as described above. The contribution of a particular isotype was expressed as the percentage of the total anti-tAChR response.

Determination of anti-AChR antibody fine specificity

The proportion of antibodies directed against the main immunogenic region (MIR) of rAChR from denervated muscle was determined in pooled sera from young and aged rats by a competitive ELISA using MoAb 35 as a reference anti-MIR antibody. Briefly, 96-well ELISA plates were coated with 50 μl MoAb 153 (15 μg/ml) for 2 h at 37°C and incubated overnight with 200 μl rAChR extract. MoAb 153 is a rat anti-AChR MoAb directed against a cytoplasmic epitope of the AChR [24]. After washing, plates were incubated with 50 μl pooled serum diluted 1:5–1:320 in P–BSA–T for 1 h at 37°C. Subsequently, 50 μl MoAb 35 coupled to horseradish peroxidase (MoAb 35–HRP; 0·1 ng/ml) were added to the wells and incubated for 1 h at 37°C. After washing, the colorimetric reaction was developed as described above. The percentage inhibition of MoAb 35–HRP was calculated as follows: ((average $OD_{450\,nm}$ of duplicate wells with MoAb 35–HRP alone – average $OD_{450\,nm}$ of duplicate wells with both serum and MoAb 35–HRP)/average $OD_{450\,nm}$ of duplicate wells with MoAb 35–HRP alone) $\times$ 100. Results are expressed as percentage inhibition of binding of MoAb 35–HRP to rAChR.

Before assaying antibodies directed against the α-BT-binding site of tAChR, IgG was isolated from sera pooled from young or aged rats by affinity chromatography on protein G Sepharose (Pharmacia). Anti-tAChR titres were determined by ELISA; to correct for differences, the IgG fraction was diluted with normal young rat IgG. Polyvinyl 96-well microtitre plates were coated with tAChR (5 μg/ml) for 1 h at 37°C and subsequently incubated with increasing concentrations (0·1–100 μg/ml) EAMG or control IgG at room temperature for 16 h. These were removed by aspiration and plates were incubated with a limiting concentration of ^{125}I-α-BT (2 pmol/ml) for 2 h at room temperature. Plates were washed and radioactivity was counted in a gamma counter. The percentage inhibition of ^{125}I-α-BT was calculated as follows: ((average ct/min of duplicate wells with ^{125}I-α-BT alone – average ct/min of duplicate wells with both serum and ^{125}I-α-BT)/average ct/min of duplicate wells with ^{125}I-α-BT alone) $\times$ 100. Results are expressed as percentage inhibition of ^{125}I-α-BT binding to tAChR.

In vitro *antigenic modulation on TE671*

The capacity of sera to induce loss of AChR by antigenic modulation was tested in resistant old and susceptible young male rats, using TE671 cultures as previously described, with minor modifications [25]. The rhabdomyosarcoma cell line TE671 expresses human nicotinic AChR [26]. TE671 cells were cultured to confluency at 37°C in 24-well tissue culture plates (Costar, Europe Ltd, Badhoevedorp, The Netherlands) in Iscove's modified

Dulbecco's medium (IMDM), supplemented with 5% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% pyruvate and 2·5 μM dexamethasone; for subsequent steps this was supplemented with 40 μg/ml cycloheximide. Serum from young and aged male rats was heated to 56°C for 1 h to inactivate complement components. Confluent cultures were incubated with 250 μl of serum dilutions (1:5–1:160) for 2 h. In order to label cell-bound AChR, 50 μl of fresh medium, containing 2·75 nM ^{125}I-α-BT, were added and incubated for 3 h. Subsequently, wells were washed twice and cell-bound radioactivity was quantified by adding 1 ml 2% SDS in PBS and counting in a gamma-counter. Background radioactivity was determined by adding 100-fold excess of unlabelled α-BT (500 nM) during the entire course of the experiment. All measurements were determined in triplicate in three separate experiments. The percentage loss of surface AChR by antigenic modulation was calculated as follows: ((ct/min in the presence of medium alone – ct/min in the presence of serum)/ct/min in the presence of medium alone) × 100.

Localization of membrane attack complex and complement regulatory proteins

Deposition of complement component C3 or C5b-9 (membrane attack complex (MAC)) was analysed in muscle biopsy cryosections using polyclonal rabbit anti-rat C3 antibodies or mouse MoAb 2A1 directed against rat C5b-9 (a kind gift of Professor W. G. Couser, Department of Medicine, University of Washington, Seattle, WA). Rabbit polyclonal antibodies against (mouse) CD55 (decay-accelerating factor (DAF)), CD59 and vitronectin were used to stain for complement regulatory proteins (a kind gift of Professor M. Daha, Department of Nephrology, University Hospital Leiden, and Dr E. de Heer, Department of Pathology, University of Leiden, The Netherlands). Muscle biopsy cryosections of young and aged male and female rats were acetone-fixed for 10 min at 4°C and air-dried for 5 min. After washing three times in PBS, the sections were incubated first in 2% P–BSA and then with the respective monoclonal or polyclonal antibodies together with rhodamine-labelled α-BT (Molecular Probes, Eugene, OR) for 1 h at room temperature. After washing with PBS, the slides were incubated for 1 h at room temperature with FITC-conjugated rabbit anti-mouse or swine anti-rabbit antibodies (Cappel, Organon Technika, Boxtel, The Netherlands).

Statistical analysis

The Wilcoxon rank test was used for statistical analysis.

RESULTS

Clinical assessment

Both young male and female rats showed mild to moderate (+ or + +) clinical signs of muscular weakness 6 and 10 weeks after primary immunization, respectively, whereas aged male and female rats were clinically resistant to induction of chronic EAMG (Table 1). Weight loss was used as a measure of EAMG severity, reflecting difficulty in eating and drinking (Fig. 1). Weight loss was significant in the young but not in the aged immunized rats ($P < 0{\cdot}05$); the PBS-immunized controls gained weight.

AChR loss from muscle

The AChR content was significantly reduced in both young and aged females ($P < 0{\cdot}05$; Fig. 2), even though muscular weakness was only observed in young animals (Table 1). Significant AChR loss ($P < 0{\cdot}05$) was seen in young male rats, but not in the older rats (Fig. 2).

Table 1. Clinical score in chronic EAMG

Chronic EAMG	Age (weeks)	Rats/group (*n*)	Clinical EAMG score			
			−	+	++	+++
Female	10	7	2	3	2	—
	122	8	8	—	—	—
Male	10	10	4	1	5	—
	128	10	10	—	—	—

Development of muscular weakness in chronic EAMG is age-related. Muscular weakness was measured 6 (female) or 10 (male) weeks after immunization with Torpedo acetylcholine receptor (tAChR). Young female and young male rats developed clear signs of muscular weakness ($P < 0{\cdot}05$). No clinical signs of disease were found in PBS/Freund's complete adjuvant (FCA)-immunized control rats.

Anti-AChR antibody titres and isotypes

Anti-rAChR antibody titres were measured at several time points after immunization in order to detect differences in the kinetics of the antibody response between young and aged animals. At 4 weeks after primary immunization both young and aged female rats were boosted with tAChR in FIA. Two weeks later, the mean anti-rAChR antibody titre in aged female rats was significantly lower ($P < 0{\cdot}01$) than in young female rats (Fig. 3a). After a single immunization, titres were somewhat lower in aged than in young rats, but increased after the former were boosted (Fig. 3b). At all stages they were considerably lower in males than in young females.

The isotype distribution of anti-tAChR antibodies in chronic EAMG was similar in both young and aged male and female BN rats (Fig. 3c). The isotype distribution of anti-rAChR antibodies

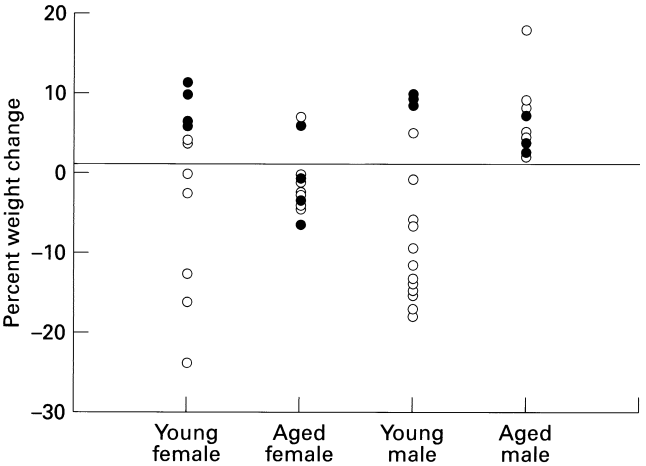


Fig. 1. Weight loss as a measure for EAMG severity. Weight loss was measured 6 (female) or 10 (male) weeks after immunization with Torpedo acetylcholine receptor (tAChR)/Freund's complete adjuvant (FCA) (○) or PBS/FCA (●). Young female and male rats showed 6·7 ± 8·1% and 8·0 ± 10·8% weight loss from the initial weight. In asymptomatic aged rats no weight loss was observed. Results are expressed as percentage weight loss from time of immunization.

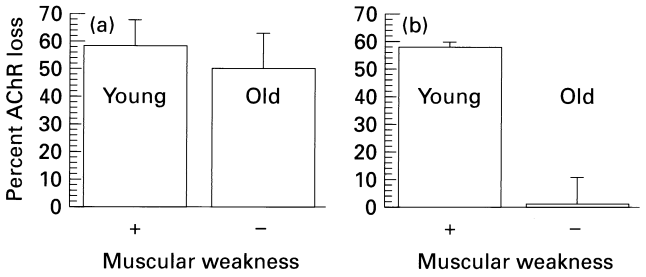


Fig. 2. Resistance to chronic EAMG is age- and sex-related. Muscular weakness and total acetylcholine receptor (AChR) concentration was measured 6 (female) (a) or 10 (male) (b) weeks after primary immunization in total muscle. Both young and aged female rats had comparable AChR loss ($58 \pm 10\%$ and $50 \pm 13\%$, respectively). Significant AChR loss ($57 \pm 2\%$) was also found in young male rats. Aged male rats were resistant to AChR loss. These results indicate that aged rats are resistant to EAMG. AChR loss is expressed as percentage of the AChR content of PBS/Freund's complete adjuvant (FCA)-immunized control rats. Each bar represents mean of five rats $\pm$ s.d.

was not determined because of technical limitations. However, since the spectrotypes of anti-tAChR antibodies and the cross-reactive anti-rAChR antibodies are identical, it is unlikely that the isotype distribution of anti-tAChR and anti-rAChR antibodies is different [27].

Anti-AChR antibody fine specificity

We tested whether differences in specificity for the MIR correlated with susceptibility. As expected (Fig. 3a,b), pooled sera from females showed higher total titres than from males, but there were no age-related differences in the proportion specific for the MIR (Fig. 4a). Clinical susceptibility might be determined by differences in antibodies interfering with binding of acetylcholine to the AChR. We determined the proportion of antibodies directed against the α-BT-binding site (Fig. 4b). The anti-α-BT-binding site titre in serum against rAChR was below detection level (data not shown). Since the structural homology between the α-BT-binding site of rAChR and tAChR is very high [28,29], we measured the inhibition of ^{125}I-α-BT binding to the α-BT-binding site of tAChR using purified IgG derived from serum of young and aged rats. Total IgG antibody levels against tAChR were about four times higher in females, regardless of age. While the inhibition of ^{125}I-α-BT binding correlated with this difference, there was again no age-related change in the proportion of these inhibitory antibodies.

In vitro *antigenic modulation*

Resistance to AChR loss in aged male rats might result from a reduced AChR degrading efficiency of the antibodies. However, decomplemented, pooled serum from young and old male rats caused similar AChR loss from cultured TE671 cells (71% and 76% at 0·1 serum dilution of young and old rats, respectively) (Fig. 5). Since aged female rats were already susceptible to AChR loss *in vivo*, the capacity of serum from these rats to induce antigenic modulation *in vitro* was not tested.

Membrane attack complex and complement regulatory proteins

Complement plays a central role in the pathogenesis of EAMG; therefore we analysed the presence of MAC and complement regulatory proteins in young and aged male and female rats 6 or 10 weeks after induction of chronic EAMG in muscle biopsy cryosections. Deposits of complement component C3 and MAC coincided with the endplate regions in both young and aged rats (Fig. 6a,b), but were not detectable in PBS-injected age-matched controls. In addition, proteins that restrict the cytolytic activity of homologous complement components, i.e. CD55, CD59 and vitronectin, were similar in endplate regions of both susceptible and resistant rats (Fig. 6c–h) and again were not seen in controls.

DISCUSSION

In this study, susceptibility to EAMG was related to age and sex. In the chronic EAMG model, induced by active immunization with tAChR, aged animals were clinically resistant to EAMG, irrespective of their sex. In contrast, young animals of both sexes were

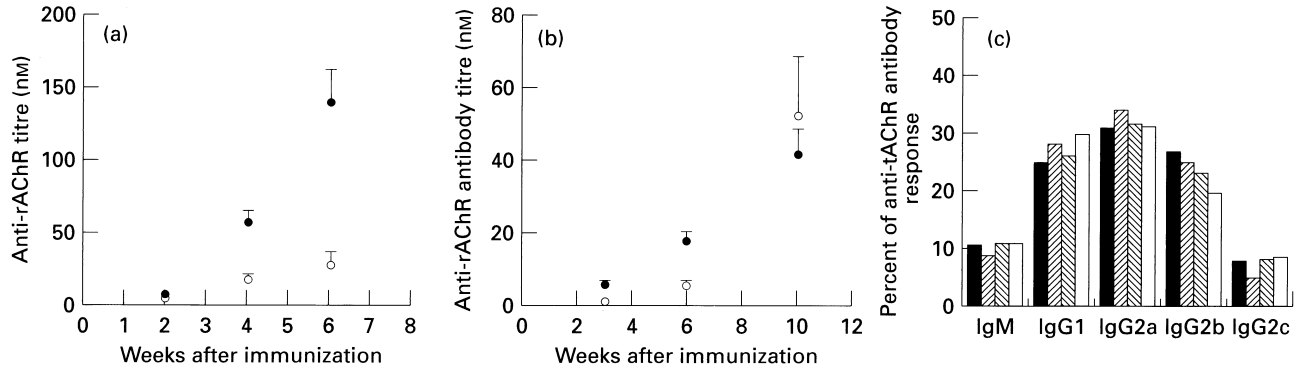


Fig. 3. Kinetics of the antibody response against rat acetylcholine receptor (rAChR) and isotype distribution of anti-tAChR antibodies. (a) Young (●) and aged (○) female rats were immunized with 10 μg/100 g body weight tAChR/Freund's complete adjuvant (FCA) and boosted 4 weeks later after primary immunization with an equal amount of tAChR/Freund's incomplete adjuvant (FIA). Anti-rAChR antibody titres were measured by radioimmunoassay (RIA) at 2, 4 and 6 weeks after primary immunization in female rats. Results are expressed as mean titres $\pm$ s.e.m. (nM), and represent at least five rats per point. (b) Young (●) and aged (○) male rats were immunized as in (a), except that only the aged male rats were boosted. There were at least five rats per point. (c) At 6 (female) or 10 (male) weeks after immunization pooled sera of young and aged male and female rats were tested for differences in isotype distribution. The isotype distribution of anti-tAChR antibodies in chronic EAMG was similar in all tested groups. The contribution of a particular isotype is expressed as percentage of the total anti-tAChR response. ■, Young male; ▨, old male; ▧, young female; □, old female.

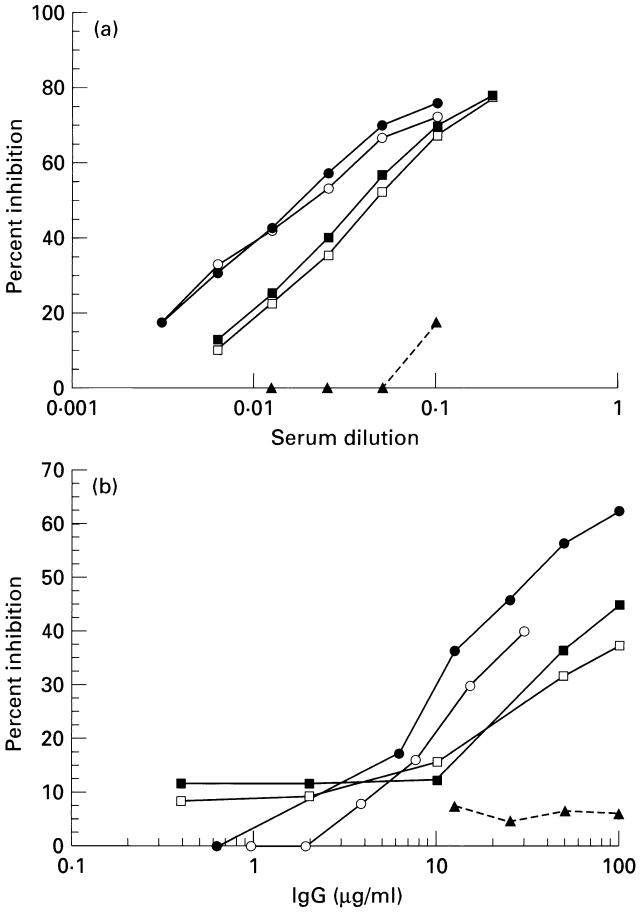


Fig. 4. Resistance to EAMG is not a result of differences in antibody fine specificity towards the main immunogenic region (MIR) or α-bungarotoxin (α-BT)-binding site. (a) The proportion of anti-acetylcholine receptor (AChR) antibodies directed against the MIR of rAChR was determined in pooled serum of young and aged male and female rats. No significant age-related differences were detected. Results are expressed as percentage inhibition of binding of MoAb 35–horseradish peroxidase (HRP) to rAChR. ●, Young female EAMG; ○, old female EAMG; ■, young male EAMG; □, old male EAMG; ▲, normal rat serum. (b) Purified IgG from pooled sera of young and aged rats was also analysed for binding to the α-BT-binding site of tAChR. No significant age-related differences were detected. Results are expressed as percentage inhibition of ^{125}I-α-BT binding to AChR. Purified IgG was used to give a higher signal-to-noise ratio. The difference between the fraction of antibodies against the MIR or α-BT-binding site between male and female rats was similar to the difference in total anti-rAChR or anti-tAChR antibody titres. IgG from control rats did not inhibit binding to the MIR or α-BT-binding site. ●, Young female EAMG; ○, old female EAMG; ■, young male EAMG; □, old male EAMG; ▲, normal rat IgG.

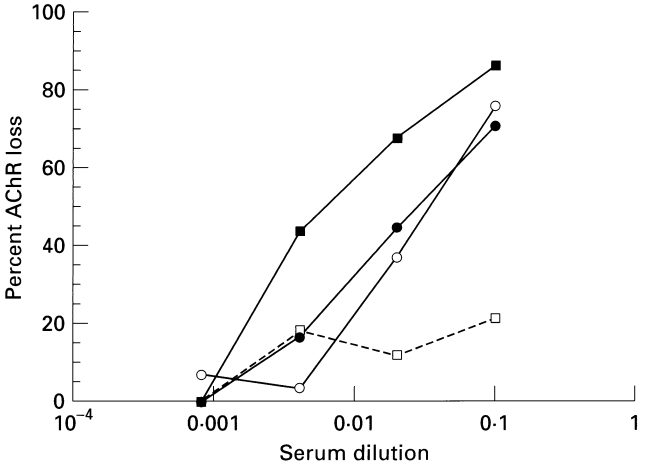


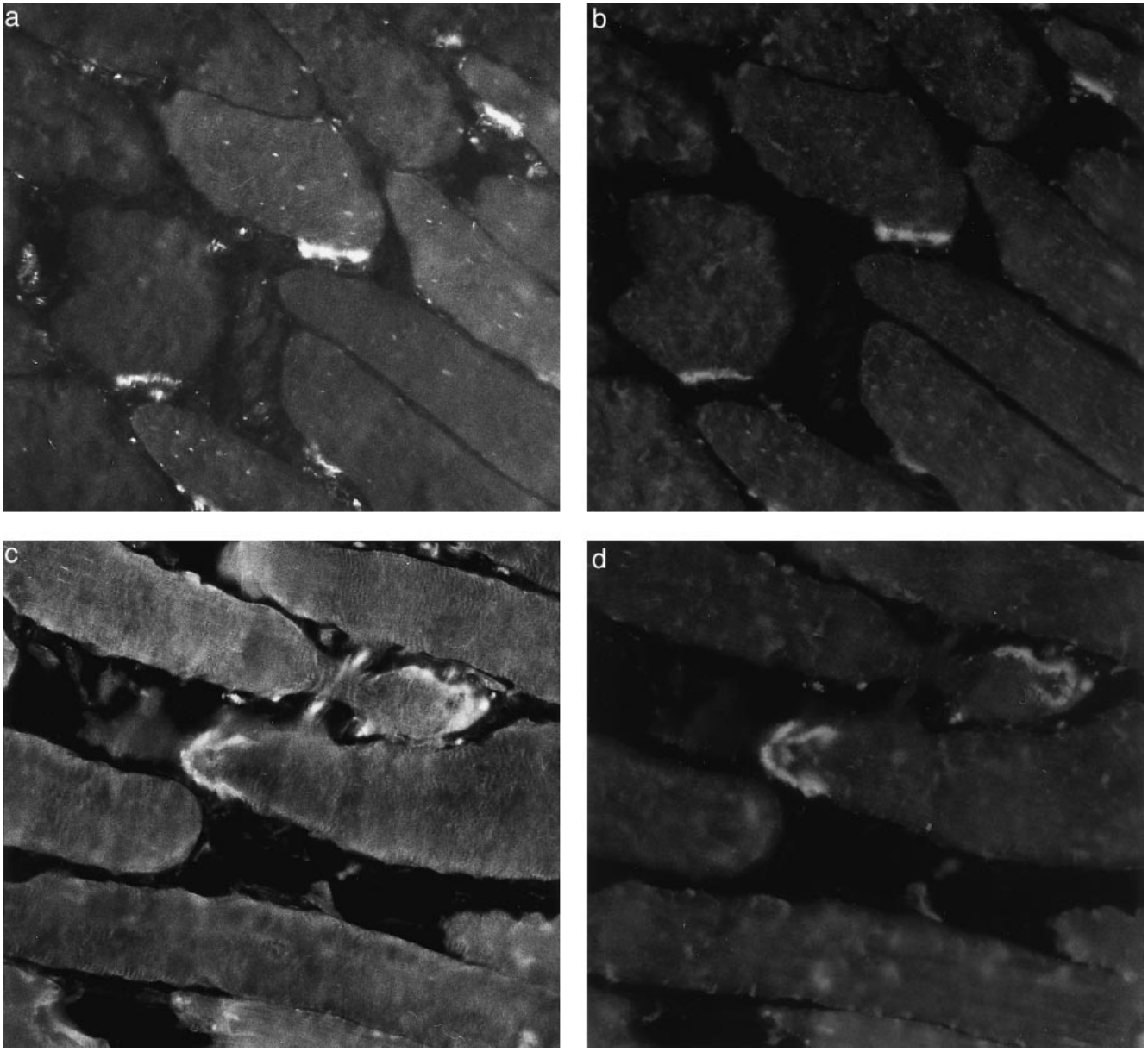
Fig. 5. Anti-acetylcholine receptor (AChR) antibodies from old resistant male rats are able to induce antigenic modulation *in vitro*. *In vitro* antigenic modulation was measured using TE671 cells and decomplemented, pooled serum from young and old male rats. Results are expressed as percentage AChR loss in the presence of serum compared with medium alone. ●, Young; ○, old; □, normal rat serum; ■, MoAb 35.

susceptible. Aged male rats were resistant to EAMG in terms of AChR loss, whereas young male rats and young and aged female rats all showed significant AChR loss. Furthermore, we have shown that resistance to chronic EAMG is not due to differences in antibodies, isotype distribution, fine specificity or to an inability to activate complement or its regulatory proteins.

Upon immunization, anti-rAChR antibody titres remained lower in aged than young female rats, as has been observed by others with responses to both autoantigens and alloantigens [12,13,30,31]. In aged male rats boosted with tAChR in FIA, anti-rAChR antibody titres were similar to young male myasthenic animals after a single injection, but they nevertheless failed to develop EAMG.

We tested for qualitative differences in the isotype distribution and fine specificity of the anti-AChR antibodies. The isotype distribution of the anti-tAChR antibody response proved to be similar in resistant and susceptible BN rats. The anti-tAChR antibody response was equally distributed among the IgG1, IgG2a and IgG2b isotype when measured by ELISA. A dominance of the IgG2a isotype response has been reported in both EAMG-sensitive Lewis and resistant Wistar Furth rats using isoelectric focusing [32]. This difference in isotype distribution might be related to strain differences, since immune sera from Lewis and BN rats show different isoelectric focusing patterns [27]. Rat IgG1, IgG2a and IgG2b subclasses are all capable of complement fixation [33]. Immunohistochemical staining revealed comparable depositions of complement component C3 and MAC in muscle biopsies that coincided with the localization of AChR in both age groups. It has been shown that myoblasts express membrane regulatory proteins in order to protect them from complement-mediated killing [34]. In both young and aged rats, the basal expression of these proteins was below the detection level. Induction of EAMG in susceptible and resistant rats resulted in comparable expression of these factors. These results suggest that resistance to EAMG is not a result of a deficiency in immunopathological effector mechanisms in aged animals, since complement deficiency results in resistance to EAMG [35,36]. Nevertheless, in aged rats the postsynaptic membrane might be more resistant to focal lysis by MAC.

Differences in antibody fine specificity between young and aged rats could correlate with a particular pathogenic anti-AChR antibody subset in the young animals inducing EAMG. A large proportion of anti-AChR antibodies in MG and rats immunized with intact AChR is directed against the MIR, located on the α-subunit at residues 67–76 [37,38]. No



differences in the proportion of serum anti-MIR antibodies were found between susceptible and resistant rats. These results are in accordance with experiments in mice in which no correlation between susceptibility to EAMG and fraction of anti-MIR antibodies was found [39].

In vitro antigenic modulation experiments with TE671 cells showed that sera from young susceptible and aged resistant male rats are able to induce similar AChR loss. These results indicate that sera from aged rats are capable of inducing antigenic modulation. This suggests that resistance to AChR loss in old male rats is not due to a deficient anti-AChR antibody response, but probably due to target organ resistance to antibody-mediated AChR loss. Nevertheless, it cannot be excluded that in aged rats the antibody heterogeneity is changed (e.g. related to fewer germinal centres) or a subset of protective antibodies is produced.

A small proportion of the anti-AChR antibodies in MG is directed against the α-BT-binding site, causing immunopharmacological blockade of the ACh binding site [40]. These blocking antibodies are lower in sera from less severely affected patients [41]. No difference in the amount of serum anti-α-BT-binding site antibodies was found between susceptible and resistant animals, indicating that the absence of muscular weakness in aged rats is not due to absence of anti-α-BT-binding site antibodies in aged rats. These results indicate that resistance to induction of EAMG is probably not a result of differences in antibody fine specificity towards the MIR or α-BT-binding site. However, we cannot exclude a deficiency of antibodies specific for the α-BT-binding site of junctional rat AChR, which is available only in very small amounts.

The induction of chronic EAMG in young female BN rats was accompanied by overt myasthenic symptoms and significant AChR

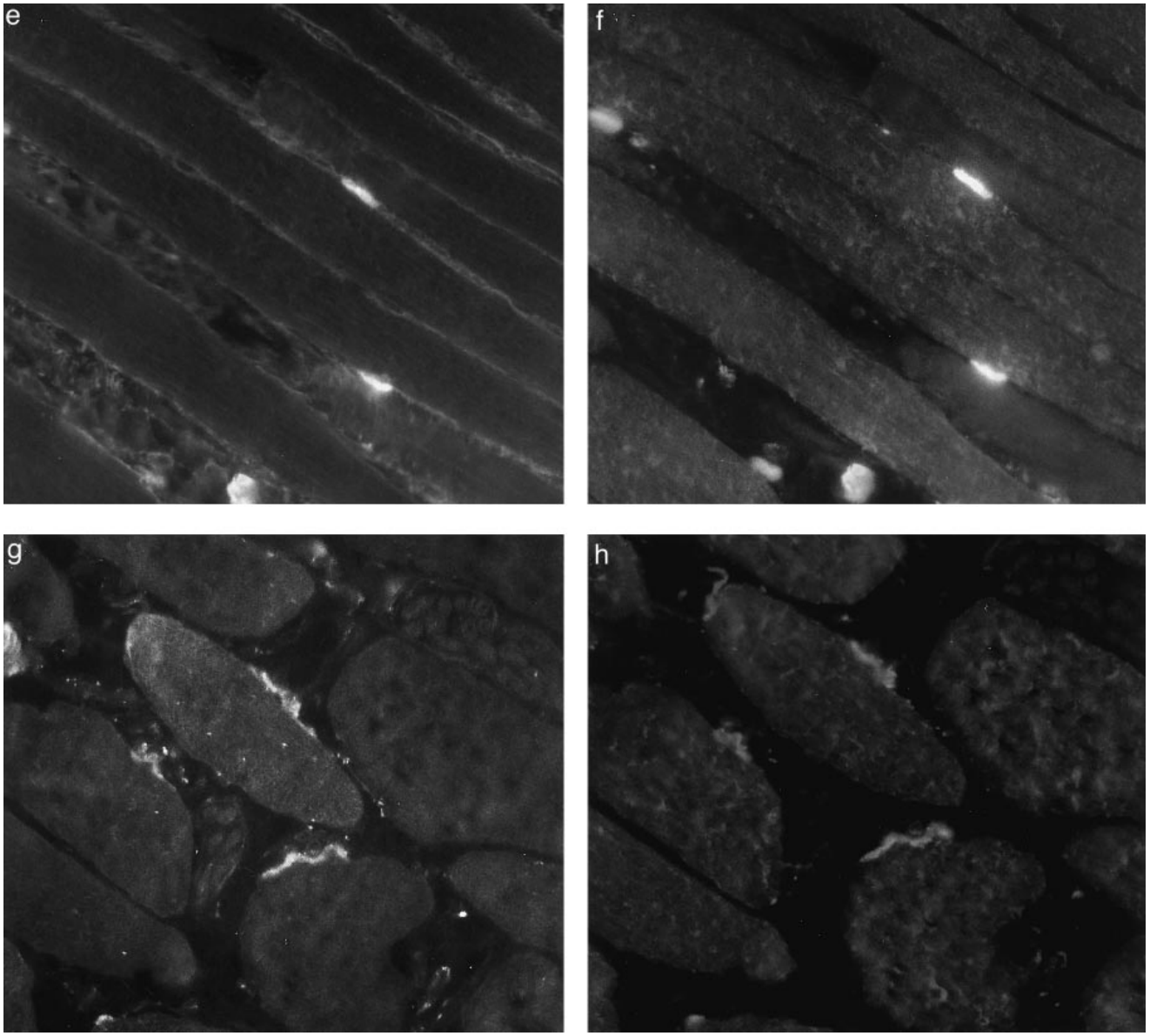


Fig. 6. Resistance to chronic EAMG is not due to an inability to activate complement or its regulatory proteins. Depositions of membrane attack complex (MAC), CD55, CD59 and vitronectin at the neuromuscular junctions were visualized by two-colour fluorescence in the same muscle section from young and aged, male and female rats 6–10 weeks after primary immunization. Shown are sections from resistant old male rats. Left photographs show muscle sections stained for MAC (a), CD55 (c), CD59 (e) and vitronectin (g). Right photographs show the same muscle sections stained for acetylcholine receptor (AChR) with rhodaminated α-bungarotoxin (α-BT) (b,d,f,h). Similar depositions were found in endplate regions of susceptible and resistant rats. No depositions of complement and its regulatory proteins were found in muscle sections of PBS-immunized control rats. Mag. $\times$ 350.

loss. In contrast, aged female BN rats showed no clinical signs of disease, although AChR loss was comparable to that in young animals. The absence of clinical signs of muscular weakness was verified by single fibre electromyography, which is a very sensitive test for the early evaluation of neuromuscular defects in EAMG [22]. No significant difference in jitter was found in aged rats before or 6 weeks after immunization (data not shown). These results indicate that aged female rats are protected against impairment of neuromuscular transmission effected by AChR loss. Aged rats may compensate for this AChR loss by changes in threshold endplate potentials (safety factors) or by increasing their quantal release more efficiently to compensate for the decreased postsynaptic sensitivity resulting from AChR loss [42]. Moreover, differences in susceptibility might be explained by changes at the endplate in aged rats, such as increased length and branching of the postsynaptic membrane and enlargement of the postsynaptic area, or wider spacing of the AChR molecules [43–46].

In contrast with the females, aged male rats did not even show AChR loss. Since resistance to chronic EAMG was not a result of deficient immunopathological mechanisms, this suggests a protective effect of androgens on the target organ in aged males compared with aged females. These results are in concordance with previous studies in experimental autoimmune models and

with epidemiological evidence in man that show that, in contrast to female sex hormones, androgens and anabolic steroids have protective effects against model diseases such as murine SLE or spontaneous autoimmune thyroiditis in obese strain chickens [18,19,47–49]. Treatment with the weakly virilizing anabolic steroid nandrolon in chronic EAMG resulted in protection from severe clinical symptoms and diminished AChR loss [50]. However, in passive transfer EAMG a deleterious effect was seen.

The results of our study indicate that resistance to AChR loss in aged male BN rats is not due to a deficiency in antibody-mediated pathogenic effector mechanisms, but more probably to resistance of the target organ. Alternatively, aged animals may have more efficient mechanisms to compensate for AChR loss. To elucidate these possible mechanisms of resistance, *in vivo* AChR degradation studies and quantification of compensatory α-subunit mRNA synthesis by competitive reverse-transcriptase polymerase chain reaction (RT-PCR) are in progress.

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