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Motor End-Plate Fine Structure of Red and White Muscles in Experimental Autoimmune Myasthenia Gravis

Akira SATOH, M.D.

The First Department of Internal Medicine, School of Medicine, Nagasaki University, 7-1 Sakamoto-machi, Nagasaki 852, JAPAN.
Tel : 0958-47-2111 (Ext 2802)

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SUMMARY

Neuromuscular junction ultrastructure in rat forelimb digit extensor muscle (FDE), extensor digitorum longus (EDL) and soleus (SOL) was quantitatively investigated in experimental autoimmune myasthenia gravis (EAMG). Experimental animals were immunized with highly purified Narke Japonica acetylcholine receptor protein plus complete Freund's adjuvant and B. pertussis vaccine : control animals received only adjuvant and vaccine. Between day 7 and 11 (acute phase) mononuclear cells infiltrated those regions of muscle where the end-plates were located and there was intense destruction of postsynaptic regions and most postsynaptic regions were splitted away from the underlying muscle fibers in FDE and EDL. In contrast, most postsynaptic folds were not detached from the underlying muscle fibers in SOL. Subsequently, between day 30 to 33 (chronic phase) morphometric analysis revealed that the postsynaptic regions of FDE and EDL were simpler and destructed more intensely than those of SOL. These results indicate that the intensity of destruction of the postsynaptic region in EAMG is apparently different between type I fibers and type II fibers both in acute phase and chronic phase.

Key Words : Experimental autoimmune myasthenia gravis (EAMG), Myasthenia gravis, Motor end-plate, Morphometry, White and red muscles

INTRODUCTION

Myasthenia gravis (MG) is caused by an autoimmune reaction to the postsynaptic

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acetylcholine receptor (AChR) protein (Patrick and Lindstrom 1973 : TOYKA *et al.* 1975 ; MITTAG *et al.* 1976 ; LINDSTROM *et al.* 1976 ; ENGEL *et al.* 1977 ; LENNON *et al.* 1978 ; SAHASHI*et al.* 1978 ; ENGEL *et al.* 1979 ; SAHASHI *et al.* 1980), and ultrastructural abnormalities of the postsynaptic region were qantitatively defined by Engel and Santa (Engel and Santa 1971 ; SANTA *et al.* 1972), and TSUJIHATA *et al.* (TSUJIHATA *et al.* 1979). Almost the same postsynaptic changes were also observed in experimental autoimmune myasthenia gravis (EAMG) (Engel et al. 1976). In human MG, these quantitative abnormalities of the postsynaptic region were the average of the abnormalitiy of both type I and type II fiber end-plates (Engel and Santa 1971 : Santa et al. 1972 ; TSUJIHATA *et al* 1979). While, in the previous paper, morphometric analysis of the motor end-plate in EAMG rats was performed on the forelimb digit extensor muscles which mainly consist of the type II fibers (ENGEL *et al.* 1976). The present study was undertaken to investigate whether the motor end-plates of both red and white muscles in EAMG rats were involved equally or ont.

METHODS

Immunization of Animals : Twelve female Lewis rats, aged 8 to 10 weeks when inoculated, were used in this study. Acetylcholine receptor protein (AChR) was solubilized from the electric organs of NARKE JAPONICA and was purified by affinity chromatography on a conjugate of cobra toxin (Miami Serpentarium Lab.) as described by EIDEFRAWI (Eldefrawi and Eldefrawi 1973). Eight animals recieved a single does of 250 pmole of highly purified AChR and complete Freund's adjuvant on the back intradermally, plus 2x10 B. pertussis organisms subcutaneously into multiple sites at the onset of the study. Four control animals received adjuvant and pertussis vaccine only. The treated animals were divided into two groups : an acute phase from day 7 to 11 after immunization and chronic phase after day 30.

Morphological study : All morphometric studies were done on the three different muscles : forelimb digit extensor muscle (FDE), extensor digitorum longus muscle (EDL) and soleus muscle (SOL). Table. 1 lists the number of animals and the number of end-plates examined by electron microscopy in each phase of the study. Morphometric analysis of the motor end-plates was carried out according to the procedure of Engel and Santa (Engel and Santa 1971 ; SANTA *et al.* 1972) as described previously and included the following : (1) nerve terminal length, in μm (NTL), (2) nerve terminal area, in square μm (NTA), (3) number of synaptic vesicles per unit terminal area, in number per square μm,

Table 1. Experimental animal. All end-plates for the morphometric analysis were transversely sectioned.

	No. of Animals	Days after Inoculation	Endplate Analyzed	
Control rats	4	32, 32, 33, 33,	SOL	34
			EDL	31
			FDE	33
EAMG rats				
Acute phase	2	7, 7,	SOL	24
			EDL	28
			EDE	30
Chronfc phas.	4	32, 32, 33, 33,	SOL	31
			EDL	45
			FDE	38
Total	10			294

(4) percentage of nerve terminal area occupied by mitochondrial profiles, (5) postsynaptic area of folds and clefts associated witn a given nerve terminal, square μm (PSA), (6) postsynaptic membrane length associated with a given nerve terminal, in μm (PSML), (7) postsynaptic membrane density derived by dividing the value of (6) by that of (5), in μm per square μm (MD), and (8) postsynaptic to presynaptic membrane ratio derived by dividing the value of (6) by that of (2) (MR). The frequencies of conformational changes occuring in control rats and EAMG rats were evaluated and the following changes were included: (1) widening of primary synaptic cleft, (2) abnormally undifferenciated postsynaptic regions (simple PS regions), and (3) absence of a nerve terminal from a postsynaptic region in one plane of sectioning (denuded PS region). In acute phase, motor end-plates were severely destroyed. Therefore, instead of morphometric analysis, we examined (1) percentage of postsynaptic regions which were detached from the muscle fiber and (2) percentage of postsynaptic regions invaded by macrophages. In addition to the electron microscopic studies, alliquots of biopsy specimens were also studied by light microscopy. Light microscopic detection of immune complexes (IgG and C3) was accomplished by the direct and indirect immunoperoxidase method. For detection of IgG, goat anti-rat IgG (Cappel Laboratories Inc., U. S. A) and peroxidase conjugated rabbit anti-goat IgG (Cappel Laboratories Inc., U. S. A) were used. For demonstration of C3, peroxidase-conjugated goat anti-rat C3 (Cappel Laboratories Inc., U. S. A.) was used. For the detection of AChR, α-bungarotoxin (Miami Serpentarium Laboratories, Miami FL) was labelled with horseradish peroxidase (Toyobo grade 3, Toyobo Inc., Osaka, Japan) by the method of Nakane

and Kawaoi (Nakane and Kawaoi 1974). All studies were done by transversely oriented sections.

Anti-AChR antibody assay : Blood samples were obtained by venesection of the tail under ether anesthesia on day 0,8,20 and 30 after inoculation. All sera were assayed for antibodies to rat denervated hind limb muscle AChR by immunoprecipitation using a modification of the previously described method (Lindstrom et al. 1976). The sera obtained on day 30 were assayed for antibodies to rat EDL and rat SOL junctional AChR by immunoprecipitation method.

RESULTS

(1) Morphological study

Control animals : None of four control animals inoculated with complete Freund's adjuvant and with pertussis organisms showed signs of muscle weakness. Light microscopic study demonstrated no inflammatory cells between the muscle fibers. There was no degenerative fibers and the cholinesterase reactive regions of the motor end-plates appear normal. Electron microscopic studies of the motor end-plates showed no quantitative abnormalities.

The acute phase : Four animals were studied. Light microscopic study showed many inflammatory cells in SOL, EDL and FDE. Many of the cholinesterase reactive sites were splitted away from the muscle fibers in EDL and FDE, but such a phenomenon was rare in SOL (Fig. 1a, 1b). Ultrastructually, the destruction of motor end-plate in FDE and EDL was more intense than that in SOL (Fig. 2a, 2b). Electron microscopic examination of the end-plates revealed that only 3 out of 30 end-plates in FDE and only 1 out of 28 end-plates in EDL were morphologically intact. In contrast, 16 out of 24 end-plates in SOL were morphologically intact (Table. 2). There was a significant difference ($p<0.05$) between SOL and EDL or FDE about the percentage of detached end-plates. No deposition of IgG or C3 was demonstrated on the residual or intact end-plates in FDE, EDL and SOL.

The chronic phase : Light microscopic studies were showed no inflammatory cells near the motor end-plates. There was no splitting away of cholinesterase reactive regions from the muscle fibers. Electron microscopic abnormalities were observed mainly in the postsynaptic regions. Results of the frequencies of confomational changes and quantitative analysis of motor end-plates are shown in table 5. In FDE and EDL, the

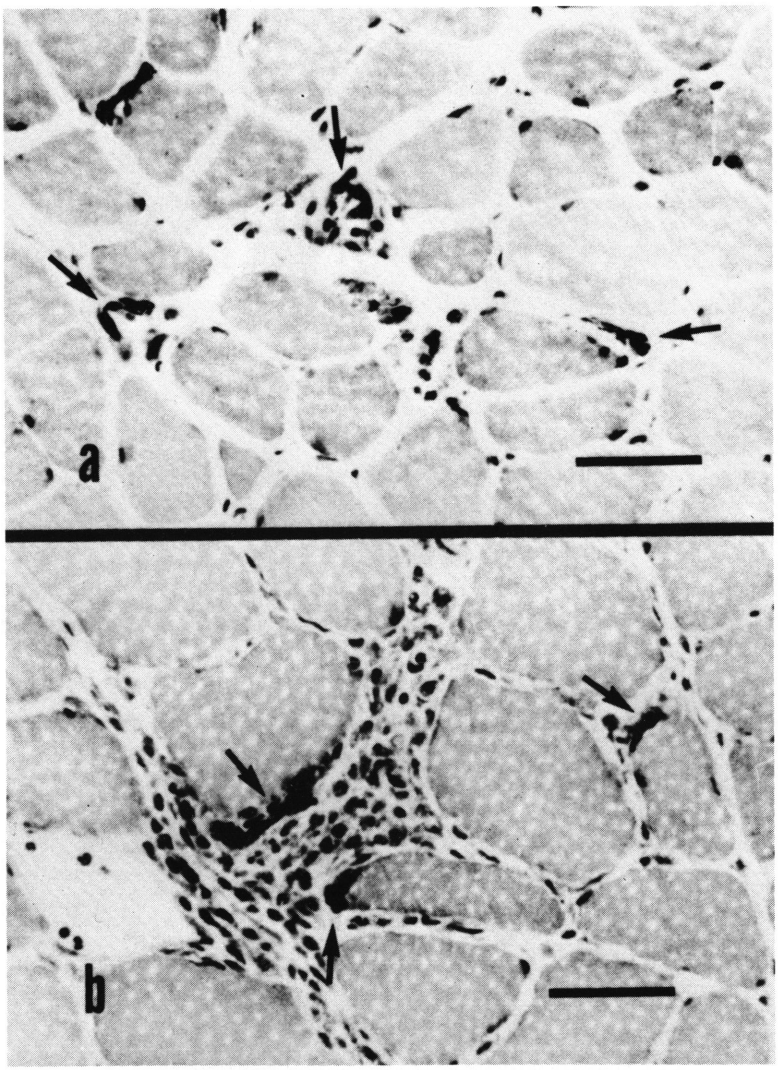

Fig. 1. Acute EAMG. Fresh frozen sections reacted for cholinesterase with acetyl thiocholine iodide and with H & E. (a) EDL: cholinesterase reactive regions (arrow) were separated from the underlying muscle fibers. (b) SOL: In contrast, the cholinesterase reactive regions (arrow) were not detached from the under lying muscle fiber in SOL. (bar = 50μm)

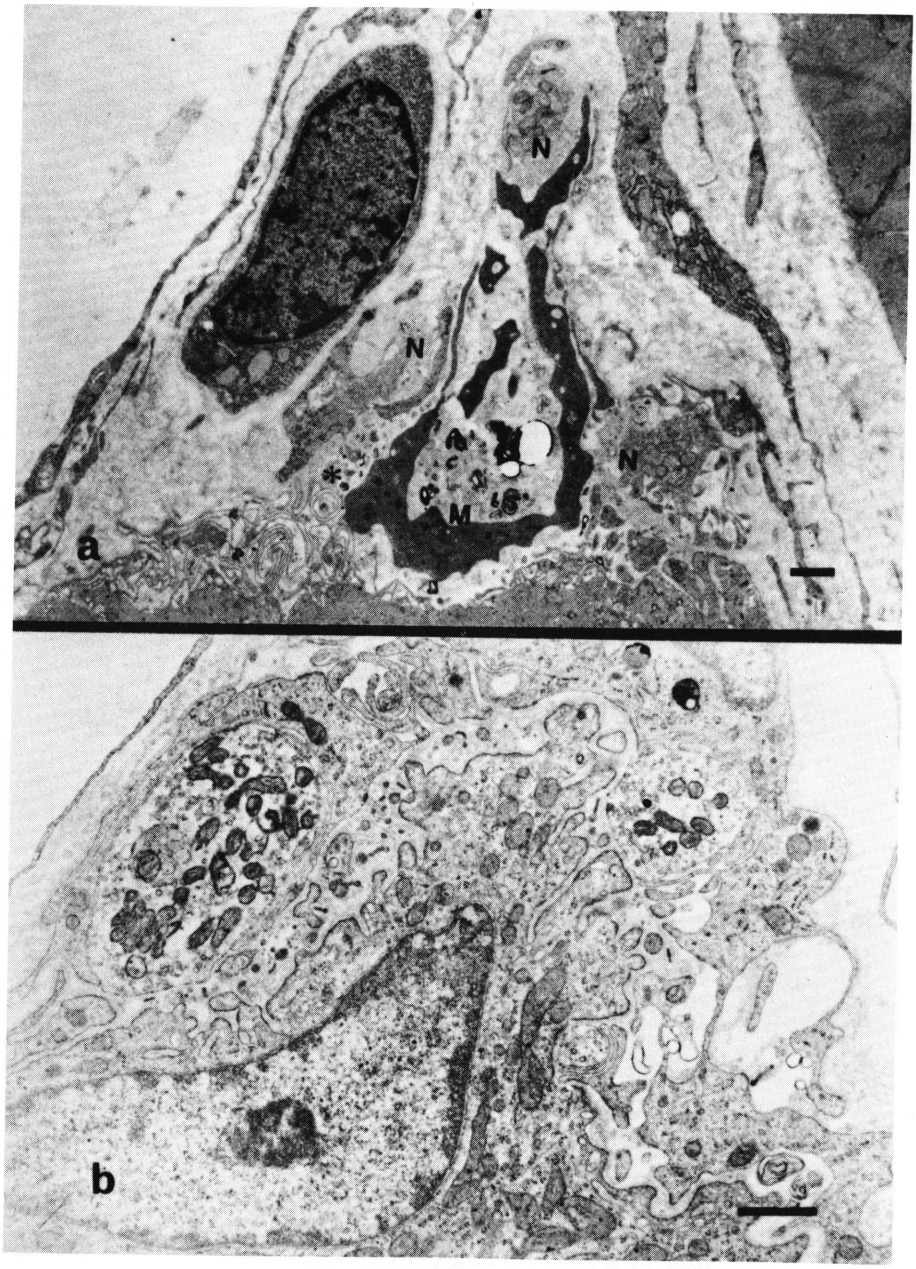

Fig. 2. Motor end-plate fine structure of acute EAMG. (a) EDL : The postsynaptic region has been remarkably destroyed. Nerve terminals (N) are separated from muscle fiber by macrophages (M) and a space containing debris and highly degenerate remnants of synaptic folds (asteric). (b) SOL : primary synaptic clefts are widened and globular residue are observed. However, the postsynaptic regions are not separated from muscle fibers and postsynaptic folds are preserved. (bar = 1μm)

Table 2. Changes of motor end-plate in acute phase: (1) percentage of postsynaptic regions which were detached from the muscle fiber. (2) percentage of postsynaptic regions invaded with macrophages.

	Detachment	Invasion of macrophage
FDE	27/30 (90%) *	22/30 (73%) **
EDL	27/28 (96%) *	20/28 (71%) **
SOL	8/24 (28%)	9/24 (33%)

* Significantly different ($p<0.001$)
** Significantly different ($p<0.05$)

postsynaptic folds were highly simplified or immature, and synaptic clefts were widened remarkably. Globular residues were observed in the widened synaptic spaces. In SOL, the postsynaptic folds were well preserved and synaptic clefts were not widened (Fig. 3a, 3b). As shown in table 5, we observed no simple PS region, no widening of primary synaptic cleft and denuded PS reggion in control animals (FDE, EDL and SOL). In EAMG rats, 10 out of 38 end-plates (26%) in FDE and 15 out of 45 end-plates (33%) in EDL showed simple PS region. In contrast, only 1 out of 31 end-plates (3%) showed simple PS region in SOL. And 13 out of 38 end-plates (34%) in FDE and 17 out of 45 end-plates (38%) in EDL showed widening of the primary synaptic clefts. In contrast, only 3 out of 38 end-plates (8%) showed widening of the primary synaptic clefts in SOL. No denuded PS regions were observed in FDE, EDL and SOL of the EAMG rats. There was significant difference about the frequency of simple ps region and the frequency of the widening of the primary synaptic cleft between FDE and SOL ($p<0.025$) and between EDL and SOL ($p<0.005$) (Chi-Square test). The deposition of IgG and C3 at the motor end-plate (Fig. 4a, 4b, 4c, 4d) and the reduction of AChR at the motor end-plate (Fig. 5a, 5b, 5c, 5d) were almost the same intensity in SOL, EDL and FDE.

Morphometric Analysis of the Presynaptic Regions: As shown in table. 3, there was no significant difference between control rats and chronic EAMG rats in SOL, EDL and FDE (Student-t test).

Morphometric Analysis of the Postsynaptic Regions: Inspection of table, 4 shows that in SOL overall means for control and chronic EAMG rats were very similar. In contrast, in EDL as well as in FDE, the PSA, PSML, MD and MR of the chronic EAMG rats were significantly decreased as compared with those of the control rats. Fig. 6 shows

Table 3. Morphometric data : Presynaptic regions. (Mean ± S. E.)

		Prespnaptic membrane length(μm)	Nerve terminal area(μm^2)	Vesicles (No/μm^2)	Mitochondrial area(%)
FDE	Control (N=33)	3.35±0.32	2.58±0.42	56.56±2.35	20.47±1.96
	EAMG (N=32)	3.16±0.32	2.55±0.33	58.33±3.63	19.49±2.07
EDL	Control (N=34)	3.11±0.20	2.47±0.35	60.44±3.11	23.48±2.36
	EAMG (N=45)	2.80±0.19	2.35±0.28	63.45±3.10	21.75±1.76
SOL	Control (N=31)	3.55±0.31	2.88±0.33	61.35±3.19	24.26±2.63
	EAMG (N=30)	3.37±0.31	2.76±0.40	59.18±4.81	21.56±2.53

* Significantly different ($p<0.001$)
** Significantly different ($p<0.01$)
*** Significantly different ($p<0.05$)

Table 4. Morphometric data : Postsynaptic regions. (Mean ± S. E.)

		Postsynaptic area per nervc terminal(μm^2)	Membrane length (μm)	Membrane density ($\mu m/\mu m^2$)	Membrane length ratio
EDE	Control (N=33)	6.28±0.39	46.00±2.88	6.83±0.20	13.32±0.91
	EAMC (N=28)	4.23±0.39	18.93±1.87	4.54±0.19	6.97±0.79
EDL	Control (N=34)	6.23±0.35	38.04±2.24	6.95±0.19	13.64±1.11
	EAMG (N=20)	3.33±0.25	13.62±1.32	4.05±0.21	5.61±0.72
SOL	Control (N=31)	6.98±0.37	42.49±2.74	6.13±0.21	14.34±1.46
	EAMG (N=30)	6.61±0.46	37.63±2.61	5.92±0.34	13489±1.56

* Significantly different ($p<0.001$)
** Significantly different ($p<0.01$)
*** Significantly different ($p<0.05$)

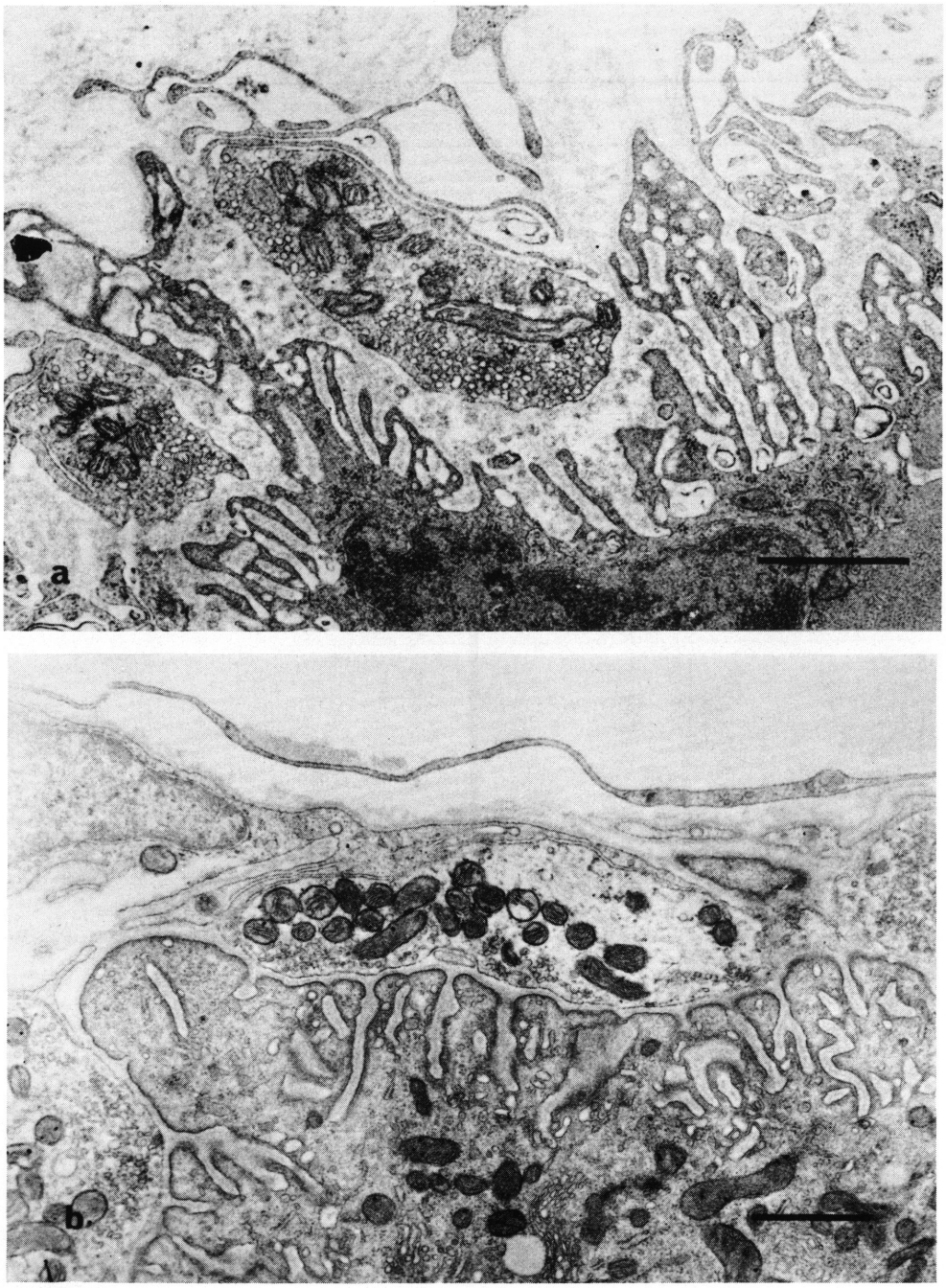

Fig. 3. Motor end-plate fine structure of chronic EAMG. (a) EDL: There is degeneration of synaptic folds with accumulation of globular residues in the widened synaptic spaces. Synaptic clefts are wider than normal. (b) SOL: Folds are well preserved. (bar = 1μm)

Table. 5. Conformational changes.

		Simple PS region	Widening of primary synaptic cleft	Denuded PS region
FDE	Control	0/33 (0%)	0/33 (0%)	0 %
	EAMG	10/38 (26%)	13/38 (34%)	0 %
EDL	Control	0/34 (0%) **	0/34 (0%) **	0 %
	EAMG	15/45 (33%)	17/45 (38%)	0 %
SOL	Control	0/31 (0%) *	0/31 (0%) *	0 %
	EAMG	1/31 (3%)	3/31 (10%)	0 %

* Signiticantly different ($p<0.005$)
** Significantly different ($p<0.025$)

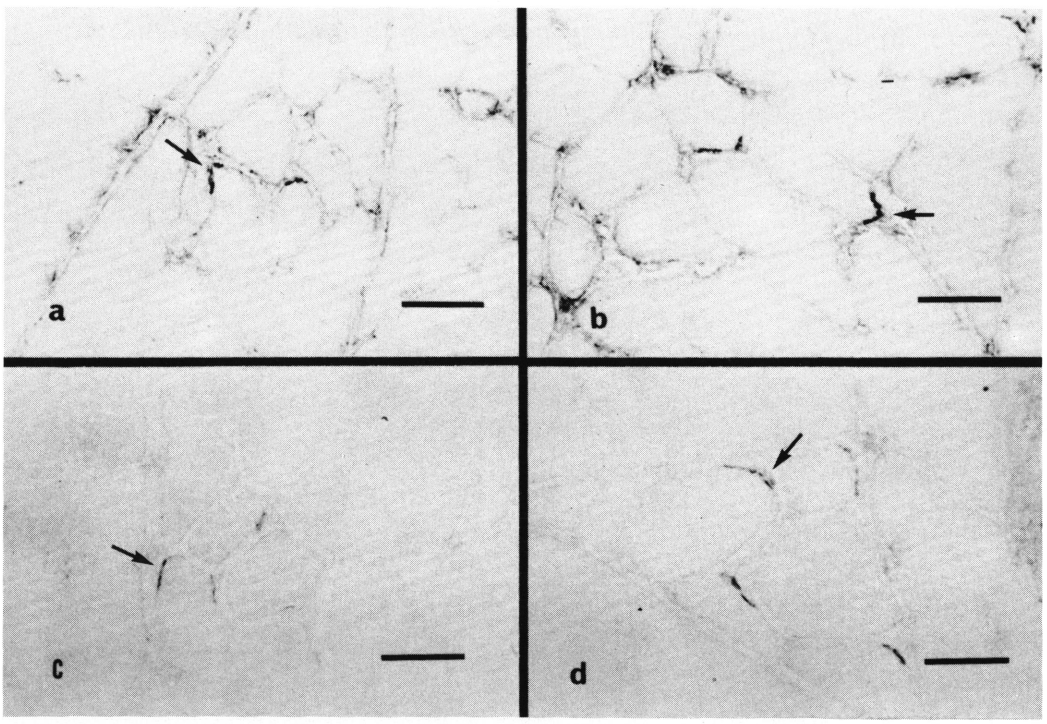

Fig. 4. Deposition of immune complexes at the motor end-plates. IgG at the motor end-plate of EDL (a) and of SOL (b), and C3 at the motor end-plate of EDL (c) and of SOL (d). The deposition of IgG and C3 at the motor end-plate was almost the same intensity in EDL and SOL. (bar = 50μm)

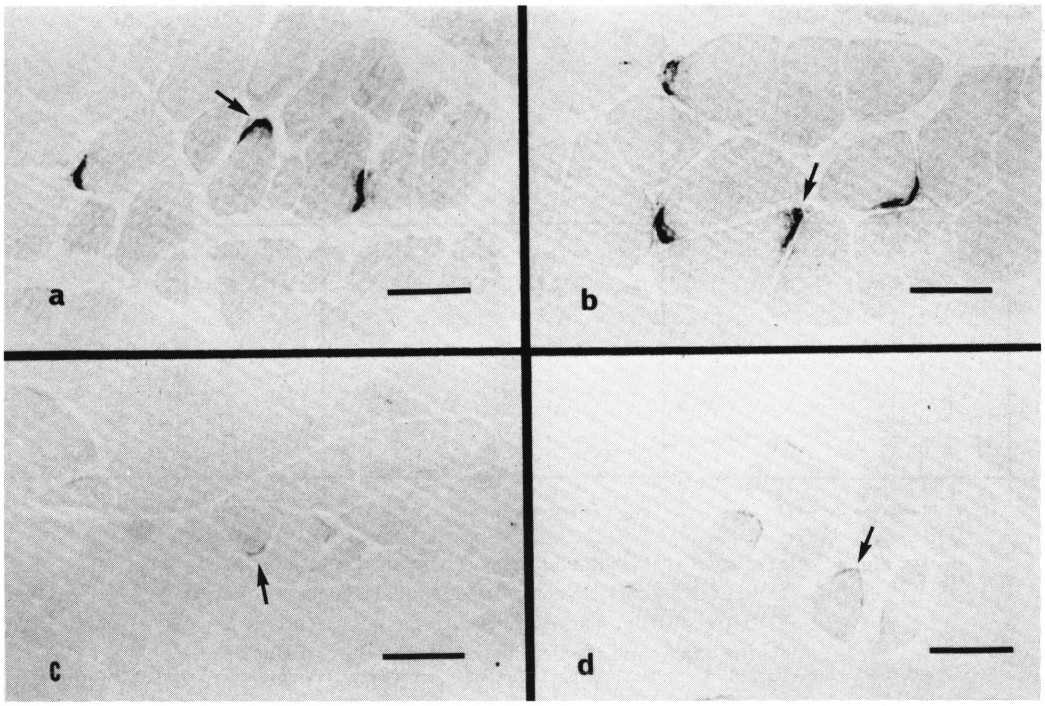

Fig. 5. Light microscopic visualization of AChR by p-BGT binding at the motor end-plate of EDL (a) and SOL (b) in control rats and of EDL (c) and SOL (d) in EAMG rats. Reduction of AChR at the motor end-plate of EAMG rats was almost the same intensity in EDL (a) and SOL (b) as compared with AChR at the motor end-plate of EDL (c) and SOL (d) of the control rats. (bar = 50μm)

the frequency distributions of the membrane density and Fig. 7 shows the frequency distributions of the membrane length ratio of SOL, EDL and FDE in cotrol and chronic EAMG rats. In EDL and FDE the distributions were sifted to the left in chronic EAMG. On the other hand, the distributions showed about the same pattern between control and chronic EAMG rats in SOL.

Titer of Anti-AChR Antibodies : We measured the titer of the blood samples on day 30. The titer of anti-denervated rat muscle AChR antibodies were 11.4 $\pm$ 3.2 on day 20 and 18.0 $\pm$ 3.8 on day 30 (mean $\pm$ SD). The titer of anti-EDL AChR antibodies was 18. 1 $\pm$ 7.9 pmole/ml and that of anti-SOL AChR antibodies was 12.5 $\pm$ 4.6 pmole/ml (mean $\pm$ SD). The titer of anti-EDL AChR antibodies was slightly higher than that of anti-SOL AChR antibodies, but there was on significant difference between these two titers of antibodies (student t-test).

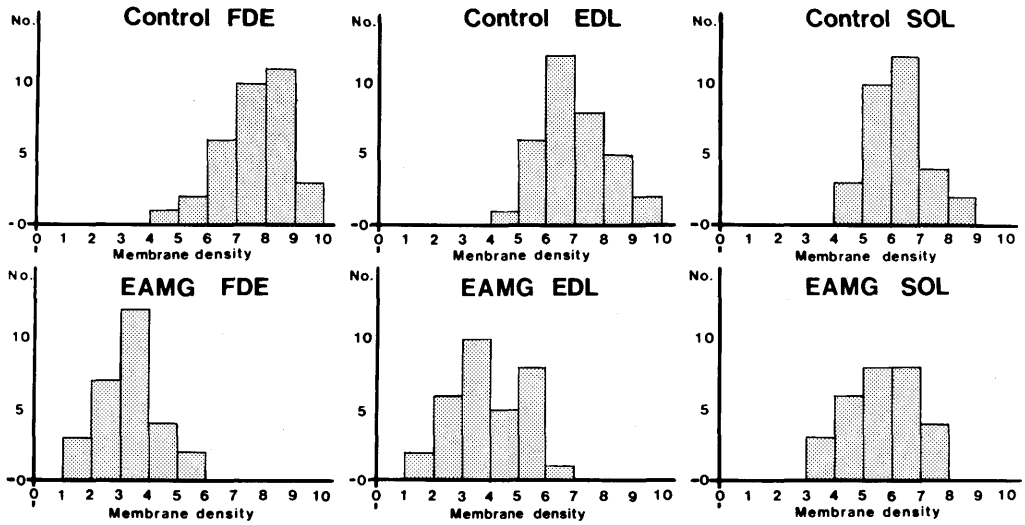

Fig. 6. Frequency distribution of the postsynaptic membrane density of FDE, EDL and SOL in control and chronic EAMG. In FDE and EDL, the distributions are shifted to the left in chronic EAMG. On the other hand, the distributions in SOL have about the same pattern between control and chronic EAMG rats in SOL.

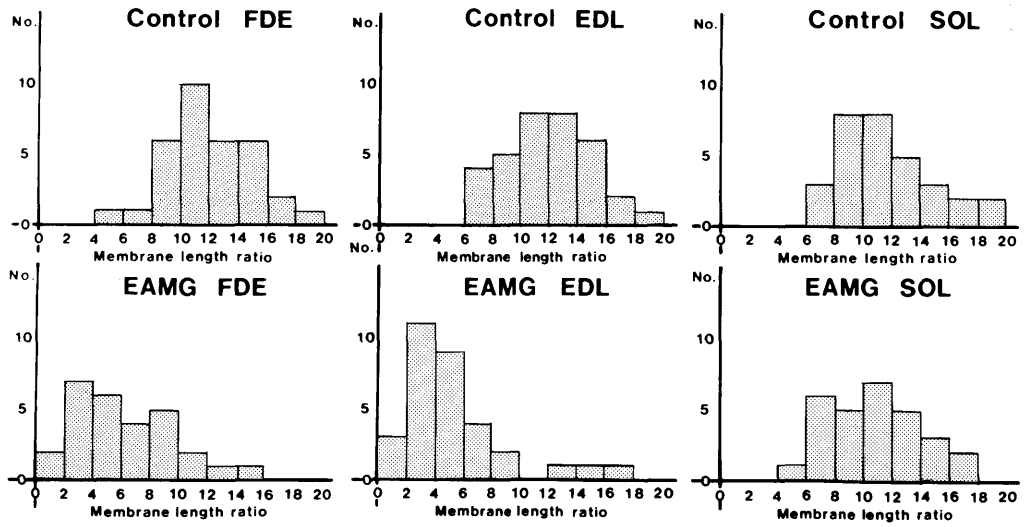

Fig. 7. Frequency distribution of the postsynaptic to presynaptic membrane ratio of FDE, EDL and SOL in control and chronic EAMG end-plate regions. In FDE and EDL, the distributions are shifted to the left in chronic EAMG. On the other hand, the distributions in SOL have about the same pattern between control and chronic EAMG rats.

DISCUSSION

The present study clearly demonstrated that the ultrastructure of the neuromuscular junction was affected in EAMG and that the postsynaptic region was the primary target of the autoimmune reaction. However, intensity of destruction of the postsynaptic region was apparently different between SOL and EDL or FDE. In EDL and FDE, there was severe destruction of the postsynaptic regions in acute phase and subsequently the end-plates were remodurated and the postsynaptic regions became simpler than normal because of continuing degeneration and regeneration. Destruction of the postsynaptic region of SOL in acute EAMG was milder than that of EDL and FDE. Subsequently, the postsynaptic regions of SOL in chronic EAMG appeared normal. These findings indicate that there is selective involvemont of muscle type of fiber type in EAMG. Engel & Santa reported that junctional abnormalities in human MG were restricted to the postsynaptic regions using intercostal mucles (SANTA *et al.* 1972) and TSUJIHATA *et al* reported that the same findings were obtained in Biceps brachii muscle (TSUJIHATA *et al.* 1979). Human intercostal muscle and Biceps brachii muscle are consisted of both histochemical type I and type II fibers. Therefore, these postsynaptic changes in human MG are made up of the changes of both type I and type II fiber end-plates. In EAMG, Engel et al described that the junctional abnormalities were confined to the postsynaptic regions using forlimb digit extensor muscle in which more than 90% are of histochemically type II (ENGEL *et al.* 1976). They reported some difference between the motor end-plate fine structure of type I and type II fibers in rat. Padykura and Gauthier demonstrated that postsynaptic folds of red muscle were shallower, more spurse, and more irregular than those of white muscle (Padykula and Gauthier 1970). Duchen documented about the same findings (Duchen 1971). HAZAMA *et al* demonstrated that the postsynaptic folds of EDL were more dense than those of SOL by morphometric analysis (HAZAMA *et al.* 1981). In this experiment, our morphometrical data of the postsynaptic regions in control rats support these previous reports. On the other hand, there are few report to have described the difference between type I fibers and type II fibers in the diseased experimental animals. Hazama et al documented that the destruction of end-plates of SOL was more intense than that of EDL in rats which were administrated ambenonium chloride for long period of time (HAZAMA *et al.* 1981). While, Duchen reported that appearance of muscular atrophy, nerve sprauting and recovery from muscular atrophy was much more rapid in SOL than in EDL (Duchen 1971) and JAWEED *et al* reported that the onset of reinnervation in SOL was earlier than that in fast

muscles (JAWEED *et al.* 1975). Engel et al reported that the rat FDE was particularly severely affected in acute phase EAMG and that in other muscles the structural integrity of the junctions and neuromuscular transmission might be well preserved, or fewer postsynaptic regions were severely affected (ENGEL *et al.* 1976). In the present study, our results clearly demonstrated that not only FDE but also EDL is much more severely affected than SOL in both acute phase and chronic phase. The reason why these selective involvement of fiber type occur is quite unknown. Here we propose some hypothesis to explain this selective involvement: (1) This difference may be related to the different antigenc character between junctional AChR protein of SOL and that of EDL and FDE. If the deposition of anti-AChR antibody is more intense at end-plates of type II fiber than at the end-plates of type I fiber, the antibody dependent-complement mediated cytolysis occurs more severely in type II fiber than in type I fiber. Recently, Levitt and Salpeter documented that the denervated neuromuscular junction contains a dual population of junctional AChRs with distinct metabolic characteristics (Levitt and Salpeter 1981). However, no one knows about the difference of antigenic character between junctional AChR of FDE, EDL and that of SOL. In this experiment, the deposition of IgG and C3 to the motor end-plate of both fiber types is about the same degree. The titer of anti-EDL AChR antibodies was slightly higher than that of anti-SOL AChR antibodies, but there was statistically no significant difference between them. Therefore, it is very difficult to explain the selective involvement of fiber type by only this hypothesis. (2) This difference depends upon the different resistance against the destruction of end-plate or the rate of recovery from destruction: In light microscopic study, the degree of the deposition of IgG and C3 at the motor end-plate of SOL is almost the same as that of EDL and FDE. Therefore, if all end-plates were attacked to the same degree and junctional AChR of both type I and II fibers had same resistance, the difference of junctional abnormality between type I and type II fiber should not be occurred. As described in the previous papers, ambenonium chrolide induced more severe changes in SOL end-plates (red muscle) than in EDL end-plates (white muscle) and botulinus toxin affected more severely EDL end-plates than SOL end-plates. Up to the present, there is no evidence that AChR protein purified from the electric organs of Narke Japonica affects fairly selectively type II motor end-plates. Judging from these studies, however, alterations of motor end-plates may depend on the different responses for the materials affecting the destruction and/or recovery of neuromuscular junctions. This point should be solved in the future. Finally, the type II fiber atrophy is not so uncommon finding in human MG (Russel 1953; Duvoitz and Brook 1973; Walton 1981). Fig. 8 shows the frequency distribution of the muscle fiber diameters

of EDL and SOL in control and chronic EAMG rat. In EDL, the distribution was shifted to the left. On the other hand, the distribution of SOL in EAMG and control was about the same pattern. Therefore, in chronic EAMG, the muscle atrophy was observed in only type II fiber, but not in type I fiber. The present study shows a possibility that type II fiber atrophy in human MG may correlate with the more selective involvement of type II motor end-plates than type I fiber end-plates (Bewan and Steinback 1977 ; Dennis 1981 ; Fambrough 1979).

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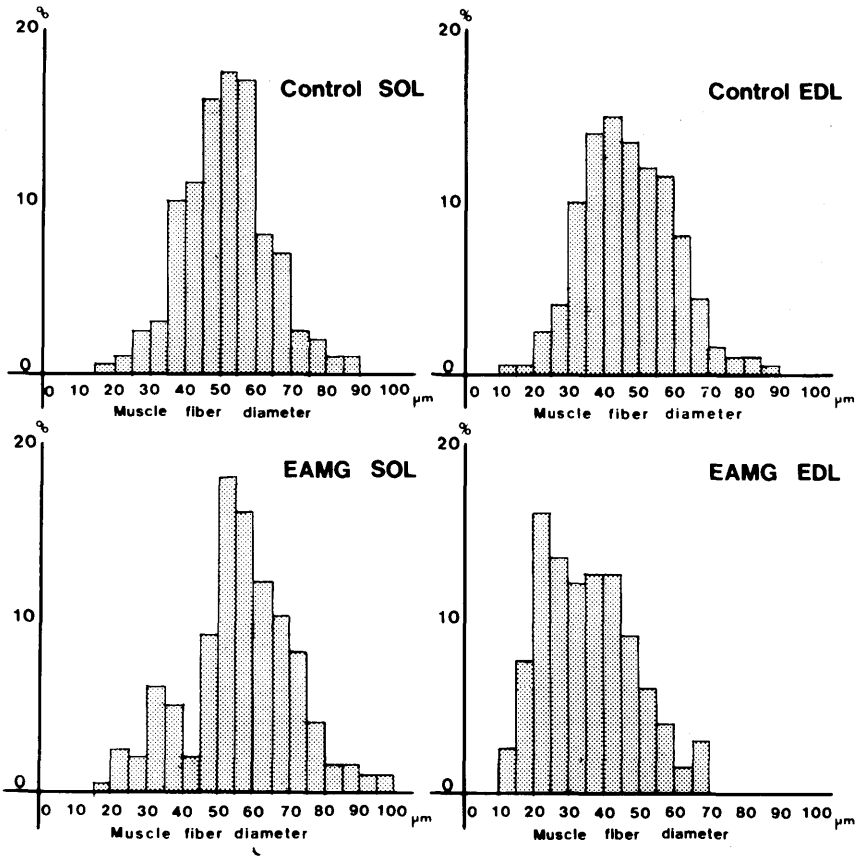

Fig. 8. Frequency distribution of muscle fiber diameter of EDL and SOL in control and chronic EAMG. The distribution in EDL is lefted to the left in chronic EAMG. On the other hand, the distribution in SOL shows about the same pattern between control and chronic EAMG.

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