FREEZE-FRACTURE, LABELLED-REPLICA, AND ELECTROPHYSIOLOGICAL STUDIES OF JUNCTIONAL FOLD DESTRUCTION IN MYASTHENIA GRAVIS AND EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS *

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INTRODUCTION

Myasthenia gravis (MG), once thought to arise from a presynaptic defect,1,2 is now generally regarded to be an “autoimmune disease”3,4 directed (pri-

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† Current addresses. The experiments described in this report were performed from 1975 to 1979 while all of the authors except Dr. Mayer and Mr. Giddings were members of the Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine.
Rash et al.: Junctional Fold Destruction

...marily) against one or more components of the post-synaptic membrane. The sequence leading to junctional fold destruction and loss of post-synaptic acetylcholine receptor (AChR) activity involves the release of anti-acetylcholine receptor antibodies (anti-AChR IgG) into the serum, the binding of IgG and complement (C3) to the crests of the folds, and the lysis of the junctional folds by the terminal components of the "complement cascade" (C9). In this study, the early events of junctional fold perforation and lysis were analyzed by freeze-fracture and conventional thin section electron microscopy, immunocytochemistry, and electro-physiological techniques. Our data reveal that the progressive loss of end plate function occurs at about the same time as a) the loss of intramembrane particles (IMPs) from the P-faces of the junctional fold crests and b) the appearance of new classes of IMP aggregates in junctional fold E-faces. In addition, we provide freeze-fracture and negative stain images of the degenerating junctional fold membranes that are compatible with those images of membranes lysed in vitro by the purified complement C5b-9 "membrane attack complex." To identify the several membrane macromolecules thought to be involved in end plate destruction, we have devised a "post-fracture labelling technique" that utilizes IgG from human MG serum for direct labelling of the intramembrane particles (IMPs) in both Torpedo electroplax vesicles and rat neuromuscular junctions (NMJ). This new post-fracture labelling technique may prove of particular value in the identification and mapping of the several complement factors that are inserted into the junctional fold membrane during membrane lysis.

Portions of this report have been described in abstract form. More detailed descriptions of end plates from additional MG and non-MG patients are in press.

MATERIALS AND METHODS

Patient Sample

For freeze-fracture and electrophysiological studies of "normal" human end plates, intercostal muscle (external layer, 5th or 6th interspace at anterior axillary line) were obtained from one female (age 53) and one male (age 63) during thoracotomy for benign pulmonary lesions. Intercostal muscle biopsies were also obtained from one man (age 33) who had adult-onset slowly progressive limb girdle muscular dystrophy (LG-MD). Because the freeze-fracture images of "normal" and LG-MD end plates were not detectably different from those of normal adult rats, end plates from these patients were treated for statistical purposes as the "non-MG" sample.

For ultrastructural and electrophysiological studies of MG end plates, intact intercostal muscles (including fragments of periosteum) were obtained from four female patients (ages 12, 26, 32, and 42 years). Their diseases varied from mild weakness of short duration (MG patients two and three) to moderate weakness of several years duration (MG patient four). Patient one, the most severely affected, was recovering from myasthenic crisis. In this report, we

† Insofar as possible, the freeze-fracture nomenclature of Branton et al. is used in this report.
concentrate our freeze-fracture efforts on patient two, who was biopsied during thymectomy at a time when she had progressing weakness. At the time of biopsy (1975), she had "autoantibodies" to single stranded DNA and double stranded RNA antigens.28

**Electrophysiology**

For electrophysiological analysis of "normal" end plates, intercostal muscles were obtained from six patients (27–49 yrs). Intercostal muscle biopsies containing uncut fibers were immersed in oxygenated Krebs-Ringers solution and allowed to equilibrate for 30–60 minutes. Bundles containing 10–30 intact fibers were dissected free. All surface fibers in the dissected bundles were monitored intracellularly at the end plate region (focal recording) using glass microelectrodes positioned under Nomarski interference optics. Resting membrane potential (RMP), amplitude of miniature end plate potentials (MEPP), frequency of MEPPs, and ACh sensitivities were determined according to published procedures.27, 28, 29 Studies of the action potentials utilized "voltage clamp" techniques as described previously.30

**Preparation of Samples for Conventional Freeze-fracture Analysis**

Samples of intercostal muscle containing intact fibers were placed in 2.5% glutaraldehyde in 0.15 M Sorensen's phosphate buffer (pH 7.2, 37°C) or in oxygenated Krebs-Ringers solution (pH 7.2, 37°C) containing 2.5% glutaraldehyde. After 5–15 minutes, the samples were chilled at 4°C and stored for 1–4 hours. Small strips containing 20–100 surface fibers were dissected free, and stained for cholinesterase activity32 by a technique modified for freeze-fracture.33 Segments enriched in end plates (approximately 0.5 mm cubes) were dissected free, slowly infiltrated with ice cold 30% glycerol, frozen in a slurry of Freon-12, and fractured and replicated in a Balzers 360M Freeze-Etch device equipped with an electron beam gun for platinum shadowing and a conventional carbon arc for replica coating. Replica thickness was regulated by a quartz crystal thin film monitor. For conventional replicas, the shadow termination frequency was 125–150 Hz. Replicas were cleaned with Clorox® bleach and chromic acid and mounted on bare 200 mesh copper grids.

**Identification of Intramembrane Particles by Post-Fracture Labelling**

*Sources of Anti-receptor IgG*

Human MG sera for post-fracture labelling experiments were obtained from four MG patients by Dr. Donald Sanders (University of Virginia Medical School, Charlottesville, Virginia. Dr. Sanders' present address is Duke University Medical School, Durham, North Carolina). Sera were fractionated at 4°C by adding an equal volume of saturated ammonium sulfate (pH 7), and centrifuged at 9,750 g for 15 minutes. The pellet was redissolved to the original serum volume in phosphate buffered saline (2 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄, and 130 mM NaCl, pH 7.4); ammonium sulfate was added to
a final concentration of 33%; and the resulting precipitate (primarily IgG) was sedimented by centrifugation. The ammonium sulfate precipitation procedure was repeated twice. The final protein solution was dialyzed against three changes of 200 volumes of phosphate buffer. After further purification by chromatography on DEAE cellulose, the final MG IgG concentration was 7–8 mg/ml. The IgG fraction was tested for interaction with purified AChR by the Ouchterlony double diffusion technique as described by Eldefrawi, et al. In contrast to sera from rabbits with experimental autoimmune MG (EAMG), the human sera do not produce precipitin lines with Triton extracted receptor protein, or block ACh or α-bungarotoxin (α-BuTX) binding to Torpedo AChR-containing vesicles (unpublished observations). Consequently, we have developed a sensitive ultrastructural assay for antibodies in human MG serum based on the binding of MG IgG to AChR-enriched Torpedo electroplax vesicles.

Ferritin-conjugated rabbit IgG directed against human IgG (Ft·R·IgG_H) was obtained from Cappel Laboratories, Downington, Pennsylvania.

Torpedo Electroplax Vesicles

For analysis of AChR binding, membrane vesicles at different levels of enrichment of AChR were obtained by homogenization of Torpedo electroplax, as previously described. For determination of binding specificity, three types of mixed vesicle preparations were used: 1) 30% of the vesicles were enriched in AChR and 70% were deficient in AChR; 2) 10% were enriched and 90% were depleted; and 3) 100% were devoid of AChR (negative control). Each preparation was labelled with human MG IgG according to published procedures. In all cases, IgG binding was proportional to the relative fraction of vesicles enriched in AChR.

Rat Neuromuscular Junctions

For post-fracture labelling of ACh receptor IMPs in rat motor end plates, extensor digitorum longus (EDL) muscles were fixed briefly (2 min.) by perfusion in situ with 0.5% formaldehyde and then rinsed immediately by perfusion with rat Ringers solution. After staining for AChE, samples greatly enriched in end plates were dissected free and prepared for freeze-fracture and post-fracture labelling.

End Plates from EAMG Rabbits

Intercostal muscle biopsies were obtained from rabbits immunized against purified Torpedo AChR. End plates obtained from rabbits showing severe flaccid paralysis were prepared for thin section electron microscopy according to the procedures used for human and rat NMJ. An in situ negative stain was used to identify damaged membranes and perforated vesicular debris within the synaptic cleft.
Unfixed *Torpedo* vesicles were either pelleted by centrifugation or were suspended in gelatin, pelleted by centrifugation and solidified by cooling to 4°C. (Chilling of unfixed membranes produces "phase separation" of lipids and proteins.\(^{38}\)) The pelleted vesicles, as well as the briefly fixed rat NMJ, were infiltrated with ice cold 30% glycerol, frozen in a slurry of Freon 12, and fractured in a Balzers 360 M device. The freshly cleaved surfaces were shadowed at a 45° angle with a very thin coat of platinum (35–65 Hz termination frequency as measured with the quartz crystal thin monitor). During early experiments using vesicles pelleted without gelatin, the replicas were stabilized by unidirectional deposition of a very thin carbon film (ca. 50–75 Å). In later post-fracture labelling experiments using gelatin embedded vesicle preparations or rat muscle end plates, the freshly cleaved surfaces were not stabilized with carbon, thereby leaving a larger portion of each IMP uncoated, and thus available for antibody binding. The samples were thawed in 30% glycerol, equilibrated to rat Ringers solution, and labelled with human MG serum or with the purified IgG fraction (1:1 or 1:10 dilution), (precedent has already been established for functional blockade of mouse AChR by human IgG.\(^{39}\)) After primary labelling, *Torpedo* vesicles were rinsed exhaustively (30 transfers through rat Ringers solution), whereas the end plate samples (35 Hz platinum layer, no carbon) were rinsed three times (in retrospect, an insufficient number of times). All samples were counter-labelled with Ft·IgG\(_{H}\) for 20–70 minutes. After labelling, samples were rinsed, post-fixed for one hour, with 1% OsO\(_4\), and either post-stained in 0.5% aqueous uranyl acetate and dehydrated in methanol series, or dehydrated in methanol series and post-stained with 3% uranyl acetate in 100% methanol. Samples were then infiltrated with plastic mixture (10% Epon 812, 20% Araldite 6005, 70% DDSA plus 1.5% DMP-30 added as catalyst), and polymerized at 70°C for 24 hours. Silver to pale gold sections were cut approximately parallel to and within the replica-tissue interface, picked up on uncoated 200 mesh grids, and stained with lead citrate.

Electron Microscopy

Sections and replicas were examined at 80 kV or 100 kV in a Siemens Elmiskop 101 high resolution electron microscope equipped with a ±24° double tilt device (goniometer). Initial magnifications were 2000× to 80,000×. All specimens were examined goniometrically at high magnification and viewed stereoscopically. High magnification images are printed as stereo pairs, which should be viewed with a conventional 2× stereoscopic viewing device.

RESULTS

Electrophysiological Analysis

Accurate placement of the tip of the recording microelectrode beneath the end plate region is facilitated using Normarski interference optics. In samples
from normal patients, all of the end plate regions located on surface fibers responded to nerve stimulation with an EPP of sufficient magnitude to elicit a muscle action potential (see Table 1). Mean MEPP amplitudes were 0.94 mV; mean MEPP frequencies were 0.33/sec, and the mean ACh sensitivity was 3573 mV/nC. In samples from MG patients two and three, 46 fibers had EPPs of sufficient amplitude to initiate action potentials (group A); 31 fibers had detectable EPPS but with amplitudes insufficient to elicit action potentials (group B); and 15 fibers had no detectable EPPS or MEPPs (group C). In fibers with detectable EPPs, MEPP amplitudes were reduced an average of 37% (group A) and 76% (group B), as compared to normal patients; mean MEPP frequencies were reduced by 22% and 48%; and ACh sensitivities by 49% and 85%. In group C (fibers with no detectable EPPs on MEPPs), ACh sensitivities were reduced by 96%. In all cases, resting membrane potentials did not differ significantly from controls (−78 to −80 mV vs. −82 mV). Since every fiber from patient 2 had diminished ACh receptor activity, we wished to determine if there were equivalent changes in the number, distribution, or cleaving face (“particle partitioning coefficient” 40) of the junctional fold IMPs. In addition, we examined the degenerating junctional fold crests for evidence compatible with proposed mechanisms of complement-mediated membrane lysis.16–18

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* (RMP), with mean amplitude of miniature end-plate potentials (MEPPs); mean MEPP frequency, and sensitivity to iontophoretically applied ACh. The present values for ACh sensitivity are approximately 20- to 100-fold greater than that originally reported for normal and MG end plates.1, 2 These significantly higher values for ACh sensitivity result from improvements in electrophysiological monitoring techniques, including focal placement of the microiontophoretic pipette using Nomarski interference optics and the ability to apply nondesensitizing pulses of ACh. Consequently, significant differences (p < .05) in ACh sensitivity of normal and MG end plates were demonstrated.

Freeze-fracture Replicas of Normal End Plates

Freeze-fracture replicas of end plates from “normal” and LG-MD patients closely resembled those from normal adult rats. The nerve terminal branches and bulb-like terminal expansion were 1–3 μm in diameter (Figure 1), and on
Figure 1. Freeze-fracture replica of portion of nerve-muscle junction from a patient with adult onset limb girdle muscular dystrophy. In this end plate, nerve and muscle membranes are indistinguishable from those of normal patients. Junctional fold P-faces (P) have 100–120 Å diameter IMPs at a density of 2000–3000/μm², whereas E-faces (E) of the junctional folds viewed in stereo (Figure 1B) are seen to be devoid of 100–120 Å IMPs. (Compare Figure 1B with Figures 4B and 4C. Figure 1A is modified from references 5 and 22.) "Railroad track arrays" (arrows) are associated with sites of transmitter release. 22, 23, 24, 25, 26, 27 Figure 1A: × 43,000, Figure 1B: × 150,000.
their P-faces, exhibited abbreviated double-doublet "railroad track arrays" of about 24–32 IMPs (small arrow). Because these minute arrays of 100 Å IMPs are located opposite the openings of the secondary synaptic clefts,26, 27 and during nerve stimulation are associated with small membrane perturbations or "dimples" (large arrowhead), the doublet arrays may be equivalent to the much larger "active zones" of amphibian end plates.41–42 Post-synaptically, the junctional folds invariably contained 100–120 Å diameter P-face IMPs at high density (ca. 3,000/µm²), usually arranged in irregular, transverse rows on the crests of the folds (P, FIGURE 1A). Thus, even the smallest fragment of junctional fold P-face (asterisk) may be recognized by its characteristic high density of large diameter IMPs. In contrast the E-face images of the junctional folds (FIGURE 1B) were devoid of IMPs larger than 100 Å and, instead, exhibited closely spaced pits.

Ultrastructural Studies of MG End Plates

In contrast to all end plates from non-MG human patients and to those from adult rats, low magnification electron micrographs of thin sections (FIGURE 2A), as well as freeze-fracture replicas (FIGURE 2B) of all end plates from MG patient 2 exhibited distinct alterations in the junctional folds. In thin section images (FIGURE 2A), many of the junctional folds were narrowed, and on their crests, had an attached layer of "fuzzy material" resolved as rods, 70 Å long and 30 Å in diameter (FIGURE 3A). Where junctional folds had degenerated, vesicular membrane debris remained, apparently held in place by the basal lamina that originally adhered to the junctional folds. In high magnification micrographs (FIGURE 3B), many of the vesicles were seen to have a thicker 100–200 Å coat composed in part of 50 × 100 Å "tufts" (large arrowhead). Often the vesicle membranes were perforated by 150–200 Å pores (FIGURE 3C) which usually had distinct sealed margins and closely associated "tufts" of material at their exterior edges (arrowhead). After in situ negative staining, similar vesicles in EAMG rabbit end plates were outlined in negative contrast (FIGURE 3D), thereby delineating the 100–200 Å pallisade layer of attached material (arrowhead). In contrast to the undamaged cell membranes, the vesicular debris did not exclude the electron dense stain, indicating either a) that the extruded vesicles had not completely resealed after membrane lysis, or b) that transmembrane lytic pores were present within each extruded vesicle. It should be noted that the nerve terminal (N) was devoid of stain, whereas the degenerating junctional folds (J) contained electron dense deposits. Thus, the membrane perforations were present only in the post-synaptic membranes and in the vesicular debris.

Freeze-fracture Images of Degenerating Junctional Folds

High magnification stereoscopic images of the crests of the junctional folds from the MG end plate illustrated in FIGURE 2B (FIGURE 4A–C) revealed that P-face IMPs were reduced from 3,000/µm² in normal end plates to as low as 1,000/µm² in recognizable junctional fold crests in MG (compare FIGURE 4A with FIGURE 1A). Conversely, E-faces (FIGURE 4B and 4C) contained greatly increased numbers of unusually large (100–150 Å) IMPs or IMP clusters
FIGURE 2. Thin section image of nerve-muscle junction from MG patient two. In this patient with MG of recent onset, most of the junctional folds were narrowed, and on their crests (see FIGURE 3A) had a layer of "fuzzy" material. A small amount of vesicular debris is observed within the synaptic clefts. (Modified from reference 8). In freeze-fracture replicas from the same patient (FIGURE 2B), the nerve terminal membrane (N) appeared normal, whereas the junctional folds exhibited distinct changes in number, size, and distribution of IMPs (see FIGURE 4A-C). Figure 2A ×28,000, FIGURE 2B ×28,000.
Likewise the extruded vesicles (FIGURE 4B) had large diameter IMPs or IMP clusters on both P- and E-faces. (Designation of E- and P-faces was based on thin section images in which the attached fuzzy layer remained on the external or convex surfaces). Other vesicular debris fragmented in a discontinuous manner (FIGURE 4A, small arrow). These perforated "vesicles" and membrane fragments (FIGURES 3D, 4A, and 7B, see below) may represent the final stages of membrane lysis. An ultrastructural model for membrane lysis (FIGURES 3E and 4D) is proposed to account for the appearance of 100–150 Å diameter E-face IMPs (FIGURES 3B and 3C), the perforated junctional folds (FIGURES 3C and 3D), and the presence of C1, C3, and C9 on degenerating junctional folds.

Binding of MG IgG to AChR in Torpedo Electroplax

It has been proposed that the 80 Å IMPs on the post-synaptic membranes of Torpedo electroplax and the 100–120 Å IMPs on vertebrate neuromuscular junctions represent polymeric AChR complexes. We and others previously have demonstrated that the anti-AChR from EAMG rabbits binds to both externally and internally exposed determinants of AChR's of Torpedo electroplax vesicles. Likewise, in this report we show that serum from some human MG patients contains IgG that is capable of binding to AChR-enriched Torpedo vesicles. IgG obtained from each of four patients with MG was incubated with Torpedo vesicle preparations that contained 10% of vesicles that were enriched in AChR and 90% of vesicles that were deficient in AChR. After exhaustive rinsing and centrifugation, the labelled-vesicle preparations were counter-labelled with FITC-IgG. The IgG from only one of the four MG patients reacted positively with the Torpedo vesicles (FIGURES 5A–C), and then only with externally exposed determinants (FIGURE 5a). At very high magnification, a 25 Å diameter branched thread (presumably the IgG-IgG bridge) was discerned linking many of the ferritin granules to the vesicle membrane. The site of IgG attachment, therefore, corresponds to an unstained antibody binding site, presumably a component of the transmembrane AChR complex. (Transmembrane proteins do not normally stain with conventional osmium-uranyl-lead stains but are stained by a ferricyanide-osmium mixture. See FIGURE 3B.)

Post-fracture Labelling of IMPs in Torpedo Electroplax

Recently we demonstrated that in "sectioned-replica" preparations of similar "sandwich-labelled" vesicles ("prefracture labelling"), the cleaving process separated the P-face IMPs from the ferritin labelled E-face pits. It was not determined at that time whether the antigenic site remained with the P-face IMPs or with an unresolved component of the pitted E-face. Therefore, the nature of the cleaving process in the immediate vicinity of the AChR proteins has been investigated using newly devised methods for post-fracture labelling of IMP components that are exposed by the cleaving process. Ultra-thin sections were obtained from the replica- "tissue" interface of Torpedo vesicles (type 2, Methods) that had been post-fracture labelled with MG-IgG. When viewed stereoscopically (FIGURE 5D), approximately 10% of the sectioned vesicles...
were found to be heavily labelled with electron dense ferritin granules, whereas in the platinum-replicated and carbon-coated vesicles less than 5% were labelled (FIGURE 5E). Stereoscopic viewing revealed that in those few replicated vesicles that were labelled, the local shadowing angle was essentially tangential to the vesicle surface. In such areas, ferritin granules were associated with only those IMPs that had been shadowed at a very low angle and coated with carbon at an oblique angle (45° to the platinum shadowing angle). In those limited areas, one side of each IMP remained uncoated by either platinum or carbon (see interpretive diagram, FIGURE 5F), and thus remained exposed and available for subsequent labelling. On the other hand, unreplicated portions of those same fractured vesicles (FIGURE 5E, large arrow), as well as intact vesicles in the adjacent thin section (see FIGURE 5D), were labelled to more than tenfold greater density than the replicated IMPs. Therefore, many antibody binding sites survive freezing and thawing (FIGURE 5D), and at least a few of these survive subsequent platinum replication and partial coating with carbon (FIGURE 5E).

Close examination reveals that only the 80–100 Å P-face IMPs were associated with ferritin granules, while areas containing only smaller IMPs (60 Å or less) were devoid of label. Furthermore, the particle-free (phase separated) areas were not labelled. Nonspecific absorption of ferritin to the exposed platinum layer was not observed and, therefore, cannot be invoked to explain the apparent specificity of binding. Finally, stereoscopic viewing reveals that most of the ferritin granules are uniformly separated from the IMPs by a distance of about 150 Å, which is equivalent to the length of two IgG molecules. Thus, these images of vesicles labelled with human MG IgG demonstrate antibody binding to IMPs of a specific size class. Moreover, the binding of human IgG to Torpedo vesicles indicates that one or more antigenic determinant(s) of the AChR complex must be shared by these distantly related species. Finally, we note that because of its exquisite sensitivity, this post-fracture labelling technique may be applicable to the identification of the molecular lesions in other autoimmune disease.

**FIGURE 3.** End-plate degeneration in recent onset MG (FIGURE 3A), chronic MG (FIGURES 3B and 3C), and in rabbit EAMG (FIGURE 3D). The crests of the junctional folds (recent onset) are uniformly covered with a “fuzzy layer” consisting of 30 × 70 Å rods, often arranged in “rosettes” (FIGURE 3A, arrowheads). In chronic MG (FIGURE 3B and 3C and 7A), the junctional folds are replaced by vesicular debris. Many of the extruded vesicles are coated with a thicker 100–120 Å coat composed of “x” and “y” shaped structures embedded in an amorphous matrix and attached to distinct transmembrane densities. (Transmembrane proteins are stained by a mixed osmium-ferricyanide reagent.) The vesicle membranes often appear discontinuous (FIGURE 3C), and in negatively stained preparations (FIGURE 3D), are shown to be perforated or incompletely sealed. An ultrastructural model is proposed (FIGURE 3E) that incorporates evidence for anti-AChR IgG bound to the junctional fold crests, complement C1-C3 attachment to the bound IgG, and membrane lysis by the C5b-9 membrane attack complex. FIGURE 3A–3D × 150,000, FIGURE 3E equivalent to ×750,000.
Post-fracture Labelling of Junctional Folds

In contrast to the labelled vesicle preparations, freeze-fractured samples of intact tissues that had been replicated with platinum only (no stabilizing coat of carbon) did not break up when thawed (FIGURE 6A). In high magnification stereo images, the replicated nerve terminal cytoplasm was seen to contain E- and P-face images of synaptic vesicles, some of which were seen to be continuous with their subjacent thin section counterparts (FIGURE 6B, small arrow). In the areas that contained replicas of the junctional fold crests, numerous ferritin granules were attached to the P-face particles (FIGURE 6B, C arrowheads), whereas very few ferritin granules were associated with junctional fold E-faces (FIGURE 6C, E). Serial section reconstruction revealed that many of the apparently "free" ferritin granules (FIGURE 6B, large arrowhead) were, in fact, associated with P-face IMPs on junctional fold crests of the succeeding thin section. In addition to the high density of Ft-IgG attached to the IMPs of the junctional fold crests, a few Ft-IgG were found attached to other areas of the replica, including areas of the nerve terminal cytoplasm (asterisks) and muscle cytoplasm (not shown). These ferritin grains may result from insufficient rinsing of samples or from clumping of secondary (Ft-IgG) labels. Before unambiguous identification of individual IMPs by Ft-IgG post-fracture labelling techniques can be claimed in complex tissues, nonspecific binding of secondary labels must be reduced.

End Plates in Chronic MG and EAMG

In more advanced stages of MG such as are seen in myasthenic crisis, most of the junctional folds are destroyed (FIGURE 7A), and in their stead, apparently held in place by the remaining deeply infolded basal lamina (arrows), are the vesicular remnants of the junctional folds. This is to be contrasted with end plate morphology in later stages of rabbit EAMG (FIGURE 7B), where initial membrane lysis (see FIGURE 3D) is superseded by extravasation of macrophage lamellae into the soleplate sarcoplasm, thereby separating and detaching the junctional folds from the remaining myofiber cytoplasm. Thus, the fulminating stage of end plate destruction in rabbit EAMG has no obvious counterpart in any of the human MG end plates examined to date. (These data are essentially confirmatory of that previously observed in rat EAMG \textsuperscript{46} and in rabbit EAMG).\textsuperscript{47}

\textbf{FIGURE 4.} High magnification stereoscopic images of junctional fold P- and E-faces from the end plate illustrated in FIGURE 2B. P-face IMPs are decreased whereas E-faces exhibit unusual 100–150 Å IMPs (arrowheads). An ultrastructural model (FIGURE 4D) presents our current interpretation of junctional fold lysis (see text). Modified from reference 15. FIGURE 4A–4C ×150,000, FIGURE 4D equivalent to ×1,000,000.
The clinical signs and symptoms of MG result from the progressive loss of functional ACh receptor molecules from the postsynaptic membrane of skeletal muscle fibers. The loss of end plate function has been correlated at the ultrastructural level with at least two phases of immunologically mediated destruction. In some patients with short term MG, anti-AChR IgG and complement factors (Clq to C3) bind to the crests of the junctional folds. Thin section images from intact junctional folds reveal a 50–70 Å layer of attached “fuzzy material” (Figure 3A). In freeze-fracture images, a change in junctional fold cleaving pattern results in a substantial decrease in the number of P-face IMPs (Figure 4A).

In the second or lytic phase, the crests of the junctional folds are progressively destroyed by IgG-activated complement factors (C5b-C9), resulting in the extrusion of perforated vesicular debris (Figures 3C and 3D) and extensive lytic damage to the remaining subsynaptic membranes (Figures 3D and 7A). In freeze-fracture images, the remaining junctional fold crests and the extruded vesicular debris have very large diameter E- and P-face IMPs or IMP aggregates (Figures 4B and 4C). The E-face IMP aggregates are consistent with freeze-etch images of red blood cell ghosts that have been lysed by complement C5b-9. Likewise, the negatively stained vesicular debris resembled images of artificial lipid vesicles that had been lysed by purified C5b-9 “membrane attack complexes.” Thus, we propose that the IMP “rosettes” in convex membrane faces of the extruded vesicles reflect the activated state of the lytic pore. We disagree with previous suggestions that the C5b-9 membrane attack complex does not penetrate the P-face of lysed membranes or that it is a “transient” pore. The rule of complementarity of membrane faces after fracturing requires that large IMPs (i.e., C5b-9 complexes, Figure 9 in ref. 18) have correspondingly large pits in the complementary membrane face. In other reports pores equivalent to those in deeply etched P-faces may have been obliterated by water vapor contamination before or during the etching process. Alternatively, the large diameter E-face IMPs in MG end plates (Figure 4C) may result from changes in particle partitioning coefficient of the junctional fold IMPs after antibody and/or complement binding. Under either circumstance, freeze-fracture replicas of the junctional fold crests of MG end plates exhibit alterations in IMP number, size, and cleaving pattern.

Figure 5. Torpedo electroplax vesicles labelled with MG IgG and examined in conventional thin sections (Figure 5A–C). Ft-IgG was found only on external vesicle surfaces (Figure 5A). At high magnification (Figure 5C), enlarged from Figure 5B, the thin antibody bridge is discerned linking the ferritin nucleus to the vesicle surface. In a post-fracture-labelled vesicle preparation examined by the sectioned replica technique (Figure 5C and 5E), both labelled (L) and unlabelled (U) vesicles are observed. Vesicles shadowed at a low angle (Figure 5E) exhibit Ft granules on the unshadowed sides of IMPs but not on the smooth phase-separated lipid-containing regions. A diagrammatic representation of the shadowing, coating, and labelling processes (Figure 5F) provides a possible explanation for the mode of access of labels on tangentially shadowed vesicles. Figure 5A: ×150,000, Figure 5B: ×150,000, Figure 5C: ×300,000, Figure 5D: ×75,000: Figure 5E: ×150,000, Figure 5F: equivalent to ×750,000.
Figure 6. Stereoscopic images of rat NMJ post-fracture labelled with Ft-IgG and examined by the sectioned-replica technique. At high magnification (Figures 6B and 6C), junctional fold crests P-faces are heavily labelled with Ft (arrowheads) whereas E-faces are devoid of label. Binding of Ft-IgG to other parts of the replica was estimated to be <10% of the density over junctional fold P-faces. Figure 6A: ×4,000, Figure 6B: ×120,000, Figure 6C: ×80,000.
FIGURE 7. A comparison of chronic MG and rabbit EAMG end plates. In MG (FIGURE 7A), junctional folds (J) often are obliterated and are replaced by junctional fold debris that is held in place by the remaining infoldings of the basal lamina (arrows). In contrast, the end plate regions (EP) of rabbit EAMG myofibers are separated from the muscle sarcoplasm by highly convoluted membrane whorls (W) which arise in part from invading macrophages (M). (See reference 47.) FIGURE 7A: ×26,000, FIGURE 7B: ×5,700.
consistent with proposed models of junctional fold destruction by immunological processes.

The serum from one of four MG patients contained IgG that bound to AChR enriched vesicles from Torpedo. The MG IgG provided an effective label for Torpedo electroplax vesicles in thin section and in freeze-fracture replicas (Figure 5). However, the IgG from that patient bound only to externally exposed determinants (Figure 5A) whereas EAMG IgG binds to both external and internal determinants. \(^{44, 45}\) When counter-labelled with \(\text{Ft} \cdot \text{IgG}_{\text{H}}\), individual MG IgG molecules could be identified and their site of binding localized to within ±20 Å in conventional thin sections (Figure 5C). "Post-fracture labelling" with MG IgG was restricted to a single class of 80 Å P-face IMPS in isolated Torpedo vesicles (Figure 5E). Similarly, the MG IgG labelled only the 100–120 Å P-face IMPS on the crests of the junctional folds of rat end plates (Figure 6B and 6C). Accordingly, we suggest that immunologically active sites are recovered intact with P-face IMPS and that many of the IgG binding sites survive freezing, fracturing, replication, and thawing.

In an attempt to correlate our freeze-fracture immunocytochemical, and electrophysiological data with data from other laboratories, we are proposing an ultrastructural model (Figures 3E and 4D) for the events leading to junctional fold lysis in MG: 1) MG IgG binds to one or more of the immunological determinants on the junctional fold crests \(^{12, 13, 21}\) (Figure 3A); 2) Complement factors Clq-3 bind to and stabilized the attached IgG \(^{12, 13, 21, 30-32}\) (Figures 3B and 3D); 3) End-plate ACh sensitivity, \(^{7, 8}\) as well as the number of \(\alpha\)-BuTX binding sites, \(^{9}\) are reduced significantly (see Table 1); and 4) Junctional folds are lysed by the C5b-9 membrane attack complex \(^{14, 16-18}\) (Figures 3C and 3D, 4B, C and D). To test this model, we suggest that pre- and post-fracture labelling with antibodies to the various integral and peripheral determinants of the AChR \(^{32}\) may allow in situ "mapping" of the three-dimensional structure of the membrane-bound AChR. Further, post-fracture labelling with antibodies to the hydrophobic portions of the C5b-9 complex \(^{53}\) may permit positive identification of that portion of the C5b-9 complex that penetrates the junctional fold membrane during lysis in MG.

REFERENCES


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**DISCUSSION**

A. G. ENGEL: In fresh-frozen tissue, did you fix with formalin between thawing and labeling?

J. E. RASH: No, the sample was briefly fixed with formaldehyde for just a few seconds before freezing.

D. B. DRACHMAN: Does the particle constitute a single receptor or clusters of monomers?

RASH: There are approximately 3,000 particles per square micron on the crest of the junctional folds in rats and humans, but there are 18,000 to 20,000 alpha-bungarotoxin binding sites. Therefore we conclude that there are approximately eight receptor subunits or receptor molecules per intramembrane particle
visualized by the freeze-fracture method. In possible support of this notion, we can discern 8 to 10 structural subunits in the large diameter particles. In addition, the molecular weight of the particle, based on its dimensions, is between 900,000 and 2,000,000, which is equivalent to accommodate 6 to 12 ACh receptor complexes.

R. TARRAB-HAZDAI: The receptor fragment inside the membrane has about 20 amino acids, with a molecular weight of about 3,000, and we have antibodies against the 30,000 dalton fragment, part of which is outside the membrane and contains the ionophore.

RASH: You may wish to attempt post-fracture labelling of the particle with your antibodies against the hydrophobic fragment of the receptor, which may become exposed by the fracturing process. The technique may allow one to map externally exposed determinants by pre-fracture labelling, and to map normally unexposed determinants by post-fracture labelling.