

Characterization Of Antibodies To Subcellular Fractions Of
Skeletal Muscles In Patients With Myasthenia Gravis And
Autoimmune Rippling Muscle Disease

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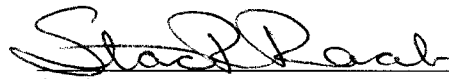
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Staci R. Raab B.S.


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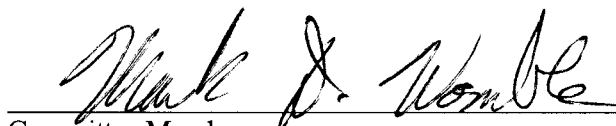

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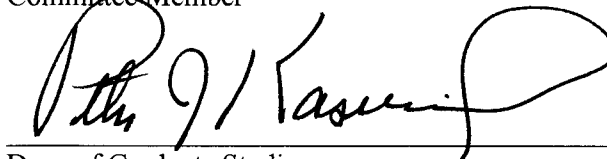
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ABSTRACT

Rippling muscle disease (RMD) is a rare autosomal dominant disease characterized by muscle weakness after resting, and electrically “silent” wave-like contractions of skeletal muscle activated by percussion or stretch. In 1995, Carl Ansevin M.D. diagnosed a patient with rippling muscle disease associated with myasthenia gravis (MG) (Ansevin and Agmanolis, 1996). This patient had no family history of RMD and experienced a disappearance of RMD symptoms after a thymectomy suggesting that RMD associated with MG may have an autoimmune origin. From western blot examination of sera from this patient and sera from other MG patients at varying severity levels (both with and without the RMD component) it was shown that MG/RMD patients displayed immunoreactivity to a skeletal muscle protein 66-97 kDa in size as well as immunoreactivity to proteins with molecular weights of 200 kDa and 300-500 kDa. Western blot analysis of MG/RMD patients’ sera displayed immunoreactivity with T-tubular membranes of rat skeletal muscle. However, this same immunoreactivity was also shown in a patient without the rippling muscle component thus indicating that these antibodies may be due to the MG, not the rippling muscle component. Sarcoplasmic reticular fractions of rat skeletal muscle did not display any immunoreactivity to MG/RMD patient sera.

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Figure 1: SEM Photo of Rat Skeletal Muscle



Introduction

This goal of this study is the identification and characterization of the autoantibodies involved with a rare autoimmune rippling muscle component sometimes associated with myasthenia gravis (MG). Since this rippling muscle component affects the skeletal muscles of patients, the structural understanding of skeletal muscles is necessary in this study. This study will deal predominantly with patients diagnosed as having myasthenia gravis with a rippling muscle component.

The muscle cells that form skeletal muscles are termed muscle fibers. It is the components of these muscle fibers, and the unique organization of these components, that is responsible for the contraction and therefore movement of skeletal muscle. Muscle fibers are large cylinder shaped cells that lie parallel to each other and are bundled together with connective tissue. These multinucleate fibers are 10-100 μm in diameter and vary in length. Each fiber is surrounded by a plasma membrane, or sarcolemma, which contains groups of myofibrils as well as the fibers' cytoplasm, termed sarcoplasm. The myofibrils found within the sarcolemma are the specialized contractile elements of the skeletal muscles and extend the length of the muscle fiber in which it is contained.

Myofibrils are each 1-2 μm in diameter and consist of an organized network of thick and thin filaments which are responsible for the contractile action of the muscles. The thick filaments range in diameter from 12 nm to 18 nm and have a length of 1.6 μm . Each thick filament is made up of approximately two hundred molecules of the contractile protein myosin. Myosin consists of two identical subunits, each having a globular head region as well as a tail region. The tail regions are intertwined with each other and are oriented toward the middle of the filament while the head portions project out in the opposite direction. This results in a bipolar structure. The head portions, or cross-bridges, of the myosin molecules contain two sites, one actin binding site and one myosin ATPase site. These cross-bridges extend out at regularly spaced intervals toward the thin filaments.

Thin filaments of the myofibrils are 5-8 nm in diameter and are 1.0 μm in length. Thin filaments consist of three contractile proteins, the major one of which is actin. Actin molecules are spherical in shape and join together to form two twisted strands creating the backbone of the thin filaments. Each molecule of actin has a myosin-binding site to facilitate binding of the myosin head from thick filaments during muscle contraction. Thin filaments are precisely organized around thick filaments to allow for

actin and myosin interaction during contraction. On a microscopic level, the arrangement of these filaments form a striated appearance consisting of light and dark bands. The dark A-bands are regions containing stacks of thick filaments which extend the entire length of the A-band. A-bands also contain portions of the thin filaments which overlap the thick filaments. The light I-bands contain the remaining portions of the thin filaments but do not contain any thick filaments. Within each A-band there is a narrow, lighter area in the center known as the H-zone which contains no thin filaments. The H-zone is also divided by the M-line which is formed by proteins that hold the thick filaments together in a vertical manner within each stack. Also present is a region of dense material within the I-bands termed the Z-line which contains cytoskeletal proteins that function to connect the thin filaments. The area between two Z-lines is referred to as the sarcomere and is the functional contractile unit of the skeletal muscle cells.

The other two proteins present in thin filaments are tropomyosin and troponin. Tropomyosin exists as a threadlike molecule which serves to conceal the myosin-binding sites on actin when the muscle is in a relaxed state. Troponin plays a role in stabilizing tropomyosin in this relaxed state but changes confirmation after the binding of calcium (which is released for muscle contraction). With calcium present, troponin allows tropomyosin to

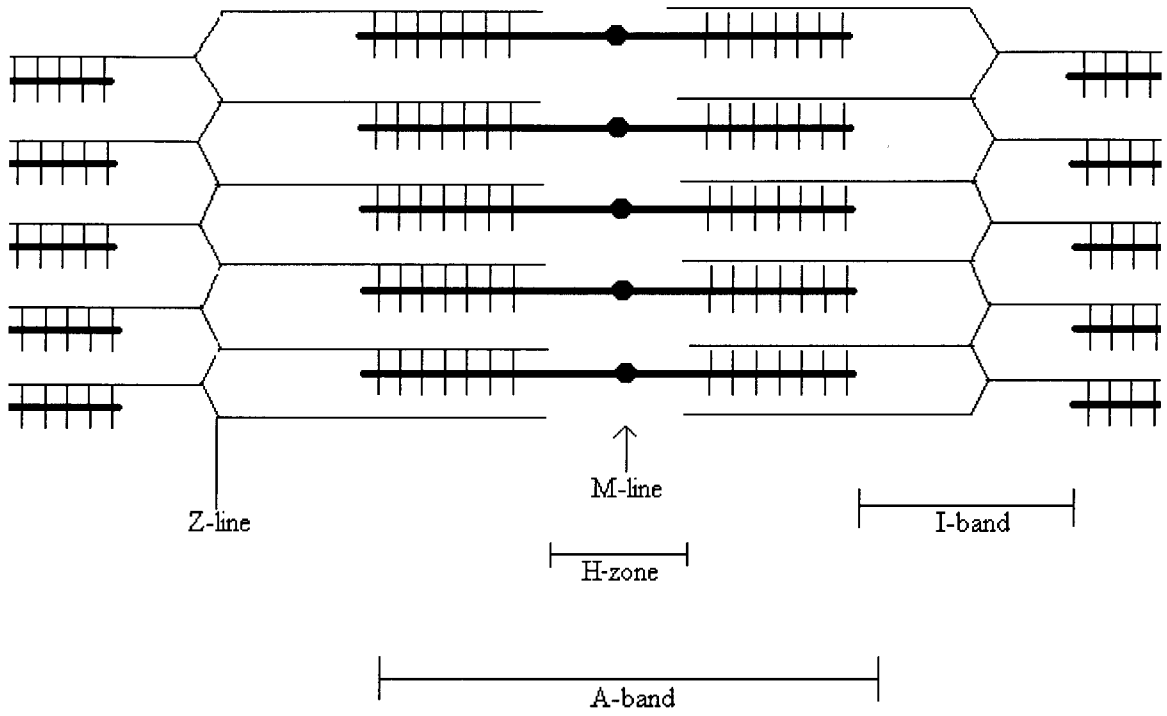


Figure 2: Skeletal Muscle Composition

slide away from the myosin-binding sites on actin, thus allowing actin to bind myosin and initiate muscle contraction.

The sarcolemma, the muscle fiber's plasma membrane, sends deep invaginations into the muscle fibers at the junctions of the A- and the I-bands forming transverse tubules (T-tubules). Closely associated with the T-tubules within the muscle fiber's cytoplasm is a membrane-bound tubular network called the sarcoplasmic reticulum (SR) which is modified endoplasmic reticulum. The SR system surrounds each myofibril and serves to store calcium when the muscle fiber is relaxed. The sarcoplasmic reticulum is not continuous throughout the entire cell. Each SR segment ends in a lateral sac which comes into close proximity to the T-tubule system. When an action potential propagates into the T-tubules, the membrane permeability of the sarcoplasmic reticulum for calcium increases greatly, therefore allowing calcium to be released into the myofibrils it surrounds.

Another type of filament present in skeletal muscle are elastic filaments. These elastic filaments are primarily comprised of the protein titin. This protein performs a stabilizing function by connecting the thick filaments to the Z lines of the muscle. Titin, also referred to as connectin, may also function to return the sarcomere to its correct resting length when

the muscle fiber relaxes (Tortora and Grabowski, 1993; Guyton and Hall, 1996).

Contraction of a skeletal muscle fiber results from a series of events that begins with the release of acetylcholine from the motor neuron innervating the muscle fiber. This release induces an action potential to propagate across the surface of the sarcolemma. The action potential also travels into the center of the muscle fiber via the T-tubule system. The action potential activates dihydropyridine receptor (DHPR), voltage-gated calcium channels, resulting in calcium release from the SR. The lateral sacs of the SR respond to the action potential by releasing stored Ca^{++} into the myofibrils via ryanodine receptor. Once the Ca^{++} enters the myofibrils, it binds to the troponin molecules of the thin filaments. A change in conformation occurs which causes the troponin-tropomyosin complex to move aside, thus allowing the myosin cross-bridges and the myosin binding sites on actin molecules to come together. Powered by ATP, the myosin head then undergoes a power stroke that pulls the thin filaments inward toward the Z-line. This movement of the thin filaments causes the sarcomere to shorten in length and is responsible for muscle contraction. Shortening continues as long as there is Ca^{++} present to keep the myosin-binding sites uncovered.

Myasthenia gravis (MG) is an autoimmune neuromuscular disorder.

MG is a disease that causes the skeletal muscles of the body to become abnormally weak and afflicts approximately 1 in 20,000 people (Guyton and Hall, 1996). Symptoms occur in an asymmetrical pattern. Some of the symptoms include problems with the extraocular muscles of the eyes, difficulty in swallowing or chewing, and slurred speech. Generalized muscle fatigue and weakness in the face, neck, arms, or legs are also common symptoms (Grob *et al.*, 1986; Aarli *et al.*, 1990). The muscles of the body are affected because MG patients produce autoantibodies to the nicotinic acetylcholine receptors at the skeletal muscle neuromuscular junction (Bartoccioni *et al.* 1980). These antibodies bind to the postsynaptic acetylcholine receptor at the motor end plate. This disturbs normal neuromuscular signal transmission by preventing the binding of the neurotransmitter acetylcholine to the receptor. The signal from the nerves to the muscle cells is greatly reduced due to these antibodies, therefore leading to the muscular weakness experienced by the patients. Antibodies to acetylcholine receptors are not the only antibodies present in MG patients. Anti-thyroid autoantibodies, as well as autoantibodies against both skeletal and heart muscle, have also been demonstrated in various studies using sera from MG patients (Beutner *et al.*, 1962; Strauss and Kemp, 1967; Penn *et*

al., 1986; Aarli *et al.*, 1990; Gautel *et al.*, 1993). Among patients that have demonstrated additional autoantibodies are those diagnosed with a thymoma, a slowly growing epithelial tumor of the thymus gland, in addition to the myasthenia gravis. Thymoma seems to accompany the MG in about 15% of patients (Williams and Lennon, 1986). These patients show a higher level of skeletal muscle antibodies than MG patients without thymoma. Penn *et al.* (1986) and Williams *et al.* (1986) used immunofluorescence to determine what portion of muscle fibers have autoantibodies against them. The striational autoantibodies found typically occur in three different patterns. Immunoreactivity occurs with A-bands only, I-band only, or with both A and I-bands in the region of the Z-line (Penn *et al.*, 1986; Williams and Lennon, 1986; Vettors, 1967). These antibodies are present in 80-95% of MG patients with thymomas and are directed against some of the muscles' main proteins, including titin, actin, myosin, tropomyosin and alpha-actinin (Aarli *et al.*, 1990; Ohta *et al.*, 1990; Pagala *et al.*, 1990; Williams and Lennon, 1986). The cause for striational autoantibody production is unknown.

Another type of autoantibody found in approximately 50% of MG patients with a thymoma are antibodies to the ryanodine receptor. The amount of antibodies for ryanodine receptor protein increases as the disease

increases in severity (Mygland *et al.*, 1994). These antibodies are not, however, found in MG patients without a thymoma.

The ryanodine receptor (RyR) is a calcium release channel protein in the sarcoplasmic reticulum that has a molecular weight between 300 and 400 kDa (Zorzato *et al.*, 1986; Lai and Messiner, 1990; Mygland *et al.*, 1994). The ryanodine receptor participates in excitation-contraction coupling by mediating the release of stored Ca^{++} from the sarcoplasmic reticulum into the muscle cell cytoplasm. Within skeletal muscle fibers, RyR Ca^{++} release channels consist of four ryanodine receptor subunits arranged in rows. Ryanodine receptors connect the T-tubules to the SR. The ryanodine receptor subunits are arranged in rows and are believed to contact the neighboring ryanodine receptors allowing all the adjacent RyR channels to open and close together.

Every other ryanodine receptor is associated with a T-tubular surface dihydropyridine receptors (DHPR). In the leading theory describing excitation-contraction coupling, depolarization of the T-tubule surface membrane is thought to cause DHPR to trigger Ca^{++} release through the associated ryanodine receptors via some type of mechanical connection between the two (Bers and Fill, 1998; Marx *et al.*, 1998; Tanabe *et al.*, 1990). Those ryanodine receptors not directly associated with a DHPR are

possibly then triggered by neighboring ryanodine receptors which are in contact with the T-tubular surface via a DHPR. This is of interest considering that some studies have demonstrated that many MG with thymoma patients have abnormalities in the excitation-contraction coupling event (Nielsen *et al.*, 1982; Coronado *et al.*, 1994; Skeie *et al.*, 1996).

Rippling muscle disease is an inherited muscular disorder first described by Torbergson (1975) in a family in which five members (out of the thirty-two studied) were afflicted with rolling muscle contractions after stretch or percussion, muscle stiffness, and mounding of the muscle after percussion. These contractions were found to be electrically silent. Other studies have also been done involving members of a different family with the same problem. Other studies have supported Torbergson in determining this disease to be an autosomal dominant disorder (Ricker *et al.*, 1989; Stephan *et al.*, 1994; Kosmorsky *et al.*, 1995; Ansevin and Agmanolis, 1996).

Rippling muscle disease was thought to be only an inherited disorder until a patient report by Ansevin (1996). This patient was different from previous cases in that neither his nine siblings nor his family history showed any signs of neuromuscular disease, leading to the conclusion that his rippling muscle disease was not a result of inheritance. The patient was seen

at the age of 56 in 1990 with muscle spasms and rippling muscles. He returned again in 1995 with symptoms such as muscular weakness, fatigue and autoantibodies for acetylcholine receptors which had not been present in his prior visit. This led to a MG diagnosis. The patient was treated with the immunosuppressant drugs, pyridostigmine and prednisone, and also underwent a thymoma removal after which the rippling muscle and MG symptoms disappeared. Other cases of myasthenia gravis with rippling muscles have since been reported. Since the contractions involved with rippling muscle occur after stretch and percussion but are electrically silent, it has been suggested that the contractions may result from the activation of stretch-activated or mechanosensitive ion channels (Ansevin and Agmanolis, 1996).

The existence of mechanosensitive channels in muscle tissue was demonstrated by Guharay and Sachs (1984) in studies with chick skeletal muscle. Since then, similar channels have been described in human cardiac and smooth muscle. (Kirber *et al.*, 1988; Bedard and Morris, 1992; Ruknudin *et al.*, 1993). Stretch activated channels are opened by the stretching of a muscle fiber, therefore allowing ions, including Ca^{++} , to flow into the muscle fiber. In cases of rippling muscle, this inflow of ions may be responsible for initiating the contractions observed upon percussion.

Rippling muscle patients may also have some additional abnormality that would account for their enhanced sensitivity to the effects of channel opening, which may explain why persons without the disorder do not experience percussion activated contractions.

A variety of patients' sera was made available for analysis in the present study. This has allowed for the comparison of sera from MG with rippling muscle (MG/RMD) to those from patients who have other problems related to MG (such as thymomas). The patients whose sera was used in this study have varying types of MG; one patient has MG with rippling muscles and a thymoma (we also have sera from the same patient when his symptoms later returned after treatment and a thymoma removal), two patients have MG with the rippling muscle component and no thymoma, two patients have MG with thymoma and no rippling muscles, one patient has MG without rippling muscles or thymoma and two patients have comparatively mild MG without rippling muscles or thymoma. Also included: a case of ocular MG, sera from an individual with malignant hyperthermia and sera from a healthy individual to serve as a negative control.

Results from a previous study by Watkins (1999) suggest that a number of muscle proteins are recognized by sera from MG patients.

Western blot analysis has shown immunoreactivity between muscle proteins and sera from several MG patients both with and without thymoma and the rippling muscle component. All patients showed immunoreactivity to a low molecular weight protein (66-97 kDa). Those MG patients with rippling muscles also showed reactivity to two other proteins with high (300-500 kDa) and intermediate (200 kDa) molecular weights. These were not seen on most blots using sera from MG patients without rippling muscles (see Table 1). Watkins also performed immunocytochemistry and detected striational like banding in areas that appeared to be the T-tubule portion of the myocyte. These results are shown in figure 5.

Table 1**Summary of Auto-antibody Immunoblot Patterns***

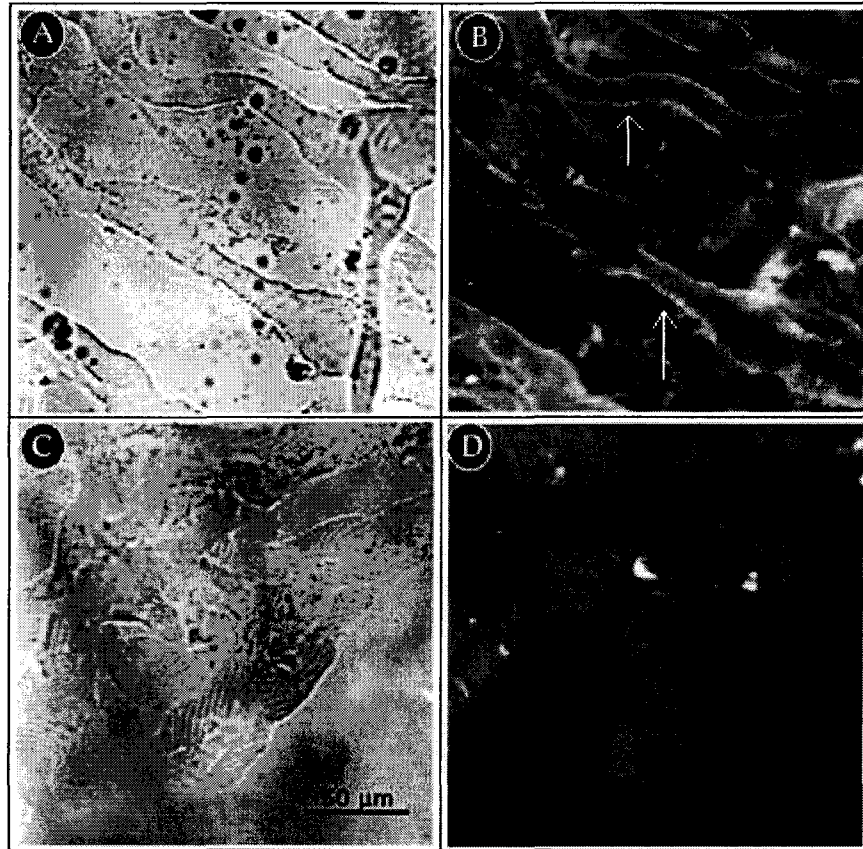
Pat. #	Patient Symptoms	Intermediate Mol. Wt	High Mol. Wt	Bands in Stacking
1	RM/MG/thymoma-no symptoms after treatment	0	+	+
1	RM/MG –symptoms returned	++	+++	+++
13	RM/MG	+/-	+	+/-
9	RM/MG	++	+++	++
3	MG / thymoma	+	+++	++
10	MG / thymoma	0	0	+/-
7	MG mild	0	0	0
6	MG	0	0	no data
12	MG mild	0	0	0
8	malignant hyperthermia	0	0	no data
2	negative control	0	0	no data
5	Ocular MG	0	0	0

*Taken with permission from Thomas Watkins' thesis "Characterization of Skeletal Muscle Antibodies in Patients with Autoimmune Rippling Muscles and Myasthenia Gravis"

Figure 3 Immunocytochemistry

Immunofluorescent micrographs of human skeletal muscle showing striational immunoreactivity (arrows). Images A and C show bright field micrographs of human skeletal muscle tissue sections. Images B and D show the same fields under fluorescence.

Figure 3 Immunocytochemistry*



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Study Aim

The goal of this study was to further identify and characterize the autoantibodies associated with the autoimmune rippling muscle component that accompanies myasthenia gravis by determining the subcellular distribution of their antigens.

Materials and Methods

Experimental Design

This study was designed to determine if autoantibodies in MG/RMD patients' sera react with T-tubular and sarcoplasmic reticular membrane proteins in fractions of skeletal muscle. In order to make this determination subcellular fractionation of skeletal muscle was performed in three different isolation procedures. A vesicle preparation was performed first to yield a fraction rich in vesicle membranes. This type of isolation did not show the immunoreactivity between the patients' sera and the high and intermediate molecular weight proteins as seen in previous data, for this reason a different isolation procedure was performed to isolate T-tubular membranes of the muscle cells specifically. Since both RyR and DHPR Ca^{++} channels are located in this area of skeletal muscle, it was hypothesized that MG/RMD patients may have autoantibodies to this portion of the muscle thus possibly playing a role in the electrically silent muscle contractions of these patients.

A third isolation was performed to isolate sarcoplasmic reticular membrane as well as T-tubular membrane. This would allow us to further distinguish whether the autoantibodies were to the SR and/or the T-tubular portion of the muscle cell. The protein composition of the different samples or fractions produced from all three isolation procedures were analyzed using SDS-PAGE and western blot analysis. SDS-PAGE was used to determine the protein concentration and composition of each fraction and western blotting was used to determine which proteins react with antibodies of MG/RMD patients. NEpHGE was also used in an attempt to further characterize autoantigens involved.

Normal rat skeletal muscle was used with MG patients' sera for analysis of any autoantibodies present. The sera were collected from patients diagnosed with MG who also demonstrated the rippling muscle component and, for comparative purposes, from several other MG patients without rippling muscles. A negative control sera from a healthy individual without MG was also available for study. Antigen specificity of the various sera were determined by SDS-PAGE or NEpHGE in combination with western blot analysis. Subcellular localization of autoantigens was performed by western blot analysis of subcellular fractions.

Vesicle Preparation

In order to prepare a membranous fraction of skeletal muscle containing T-tubular membrane, vesicles were prepared according to de Meis *et al.* (1971). Approximately 15g of rat skeletal muscle was prepared in the following manner. The muscle was minced with a razor blade then mixed with 45 ml of a cold solution containing 100 mM KCl, 2 mM EDTA, 2.5 mM KH₂PO₄ and 2.5 mM K₂HPO₄. This mix was placed into a Waring blender for 2 minutes. Next, centrifugation was performed in a Beckman centrifuge at 3,300 x g for 30 minutes to sediment myofibrils. The supernatant was centrifuged at 3,500 x g for 45 minutes to remove the mitochondria. The supernatant was then ultracentrifuged at 44,000 x g for 1 hour after which the pellet was suspended in 4.8 ml of a solution containing 1M sucrose and 50 mM KCl, sonicated to disperse the material, and centrifuged at 4,500 x g for 15 minutes. After removal of the pellet, 3.9 ml of a solution containing 2M KCl and 5mM ATP was added to the supernatant and ultracentrifuged at 80,000 x g for 90 minutes. The resulting pellet was washed twice with 10 ml of 0.1M KCl. In each washing, the material was dispersed with a sonicator and centrifuged at 80,000 x g for 60 minutes. The final pellet was suspended in 1 ml of the 0.1M KCl solution

and stored at 5°C. All steps were run at 4°C and the pellets and supernatants from each step saved for possible use in future procedures.

T-tubule Isolation

T-tubular membranes were prepared from rat skeletal muscle according to Florio *et al.* (1992). Back and hind leg rat skeletal muscle (40 grams) was used for the isolation of T-tubules for analysis. The muscle was minced with a razor blade, mixed with 50 ml of buffer #1 containing 100mM Tris, 0.3 M sucrose and placed into a blender for 30 seconds. More buffer (50 ml) was added and again blended for 30 seconds followed by the addition of buffer #2 consisting of 20mM Tris. The homogenized muscle mixture was placed on ice for 5 minutes then blended again for 30 seconds. This step was repeated seven times before centrifugation of the mixture at 3600 rpm in a Beckman J6 rotor for 20 minutes. The top layer and pellet were both removed and saved. The supernatant was filtered through a double layer of cheesecloth and centrifuged again at 10,000 x g for 20 minutes. The pellet was removed and powdered KCl was added to the supernatant to reach a final concentration of 0.5M KCl. This solution was stirred for 30 minutes on ice before being centrifuged at 150,000 x g for 45 minutes to collect a crude T-tubule pellet. The pellet was washed in buffer #2 and resuspended in this buffer at a concentration of 10 mg/ ml. The

membranes were then homogenized six times in a homogenizer and frozen for analysis in the lab.

Sarcoplasmic Reticulum and T-tubule Isolation

T-tubular and sarcoplasmic reticulum membranes were prepared according to Sabbadini and Okamoto (1983). Approximately 50g of rat skeletal muscle was prepared in the following manner. The muscle was homogenized in a Waring blender for 15 seconds every 5 minutes for an hour with 150 ml of a cold solution containing 10 mM MOPS, pH 6.8, and 10% sucrose. The pH of this mix was maintained by the occasional addition of a few drops of 5% NaOH. Next, centrifugation was performed at 15,000 x g for 20 minutes. The supernatant was then filtered through cheesecloth and ultracentrifuged at 40,000 x g for 90 minutes after which the pellet was suspended and incubated for 1 hour in a solution containing 10 mM MOPS, pH 6.8, and 0.6 M KCl. Centrifugation was performed at 15,000 x g for 20 minutes. The supernatant was ultracentrifuged for 90 minutes at 40,000 x g. The pellet was then suspended to a protein concentration of 0.35 mg/ml in an oxalate loading solution containing 20 mM MOPS, pH 6.8, 2 mM CaCl₂, 2 mM EGTA, 5 mM potassium oxalate, 80 mM KCl, 5 mM MgCl₂, and 5 mM ATP and incubated at 37°C for 10 minutes. The loading mixture was layered in 17 ml aliquots on top of a 2

layers sucrose gradient. The first layer was a 4 ml solution containing 52% sucrose and 20 mM MOPS, pH 6.8, and the second layer was a 7 ml solution containing 30% sucrose and 20 mM MOPS, pH 6.8. The gradient was then ultracentrifuged at 140,000 x g for 120 minutes. The top T-tubule layer was drawn off with a pipet, reincubated in loading solution and ultracentrifuged in the same manner previously described to further purify the fraction. The top layer was collected, washed in 50 mM imidazole, pH 7.0 and suspended in 30% sucrose, and 20 mM imidazole, pH 7.0 to final protein concentration of 1-2 mg/ml. The pellets of both centrifugations were suspended in an oxalate unloading solution of 10 mM MOPS, pH 6.8, 0.6 M KCl, 1 mM EGTA to a protein concentration of 0.2-0.35 mg/ml (determined by Bradford Assay) and incubated at 5°C for 12-18 hours. The mixture was layered in 15 ml aliquots on 4 ml of 52% sucrose, and 20 mM MOPS, pH 6.8, then ultracentrifuged at 141,000 x g for 1 hour. The top layer and the pellet were collected and used for analysis. All ultracentrifugations occurred using a Beckman anglehead rotor unless otherwise stated. All steps were run at 4°C and the pellets and supernatants from each step saved for possible use in future procedures.

Bradford Protein Assay

Bradford protein assays were used to determine the protein concentration of samples obtained during the T-tubule/ SR isolation procedure in order to dilute samples to a specific concentration as called for in the isolation procedure protocol. Five standards and one sample were all tested in triplicate. A dye solution is made with 24 parts ddH₂O and 6 parts concentrated Bio-Rad protein dye reagent. To each test tube 990 μ l of this solution is added to 10 μ l of the sample or standard being used. The five standards used include immunoglobulin or BSA at 0, 0.34, 0.68, 1.02, and 1.36 μ g/ml. Each sample's optical density was read using a Beckman spectrophotometer at 595 λ visible light and the data obtained analyzed on a Cricket graph in order to obtain information allowing for the calculation of the unknown sample's protein concentration. Figure 4 is a sample Cricket graph.

SDS-PAGE

Protein composition was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This was used to analyze the various proteins present in samples from each step of the previously described isolation procedures. The purpose of SDS-PAGE is the separation of proteins through an acrylamide gel matrix based on molecular weight.

During SDS-PAGE two types of acrylamide gels are responsible for the protein separation, resolving gel and stacking gel. The main sieving gel, or resolving gel, is poured in sets of 10-12 and vary in acrylamide concentration depending on the degree of separation required. Typically in this study 10%, 7.5% and 5% gels were used.

The 10% gels were used to initially analyze the overall protein concentration of each sample in order to best determine the amount of sample needed to load into gels for western blotting. The 7.5% and 5% gels were used during western blotting to allow very large proteins normally caught in the stacking gel portion of the gel to diffuse down into the resolving gel (the lower the acrylamide concentration, the better able proteins are to move through the gels). When SDS-PAGE is performed, a layer of stacking gel is poured ovetop of the resolving gel and functions to concentrate the samples before diffusion into the resolving portion of the gel. A comb is then placed in the stacking gel after it is layered over the resolving gel. The stacking gel polymerizes and the comb removed to form wells into which the samples can be loaded.

Each sample was prepared for electrophoresis by adding sample buffer and was then placed into boiling water for 1 minute. Between 5-10 μ l of each sample (depending on its' protein concentration) was then loaded

into the stacking gel and the gels ran for 90 minutes at a constant current of 0.025 amps. The SDS added to the samples serves to impart a negative charge on the proteins within the sample. A negative electrode is placed at the top portion of the gel and a positive electrode at the bottom. Since the proteins have a negative charge due to the SDS, they move through the gel matrix toward the positive electrode, the smaller proteins moving faster than the larger allowing the separation by size. Gels were then either stained with coomassie blue or the proteins transferred to nitrocellulose sheets for further analysis by western blotting.

Proteins were transferred from the polyacrylamide gels to a nitrocellulose sheet by placing them into a "Genie" tray from Idea Scientific in the following manner. An electrode and bubble screen were placed first on the bottom of the plexiglass tray followed by a Scotch Brit pad and sheet of filter paper. Next, the gel is placed over the filter paper followed by a nitrocellulose sheet, a sheet filter paper, a Scotch Brit pad, bubble screen, electrode, and another sheet of plexiglass is added after the chamber is filled with transfer buffer. The materials are carefully layered into the chamber to ensure that air bubbles do not get trapped between the gel and transfer membrane. The transfers were then run for 45 minutes at 25 volts. After the

transfers are complete, the transfer membranes are removed from the chamber for western blotting.

Western Blot Analysis

Immunoreactivity between patients' sera and proteins in subcellular fractions of skeletal muscle was determined by western blotting techniques. Antibodies were also used during western blot analysis to determine the composition of samples. The blots were performed by exposing the nitrocellulose sheets, obtained after transferring proteins from an SDS-PAGE gel, to a blocking buffer for 60 minutes to eliminate nonspecific binding sites. Blocking buffer contains 10% non-fat dry milk in TBS-T. TBS-T (Tris buffered saline solution containing Tween-20 detergent) is made with 0.5 M NaCl, 20 mM Tris (hydroxymethylaminomethane), and 2% Tween 20. After several rinses in TBS-T, the blot paper was placed for 60 minutes into a 1% milk solution containing patient's sera at a dilution of 1:1000. The blotting paper was removed, washed several times with TBS-T to remove primary antibody, and then placed in a 1% milk solution containing 3 μ l of secondary horseradish peroxidase conjugated goat anti-human antibody for an additional 60 minutes. Upon removal, the paper was rinsed twice with TBS-T for 10 minutes each and then twice with TBS (Tris buffered saline without the addition of Tween 20). The blots were

developed using a chemiluminescent method (PIERCE Super Signal ULTRA substrate) with autoradiography. The presence of specific marker proteins in samples was detected in the same manner using commercial antibodies anti-RyR and anti-dystrophin (Chemicon Inc.) , anti-AchR (Serotec) and anti-DHPR, specific for the α subunit, (Upstate Biotechnology Inc.) as primary antibodies and it's corresponding secondary antibody.

NEpHGE

Samples requiring additional analysis were examined using NEpHGE (non equilibrium pH gel electrophoresis), a two dimensional form of gel electrophoresis. NEpHGE separates proteins based on electrical charge and molecular weight as opposed to SDS-PAGE which separates based on molecular weight alone. Samples which showed immunoreactivity in western blot analysis after SDS-PAGE were freeze-dried overnight and added to lysis buffer to prepare the sample for electrophoresis. Tube gels were the first dimension of NEpHGE. After 20 μ l of sample is pipetted into the tube gels, they were run for 1600-2000 volt/hours and then removed from the tubes intact. Once removed from the tubes, the gels were referred to as worms. The second dimension of the procedure was performed by the placement of one worm across the top of an SDS-PAGE resolving gel and anchored into place with a 3% solution of agarose and run again at .025

amps constant current for 90 minutes. These gels were also transferred onto nitrocellulose paper for further analysis by western blotting, as described above.

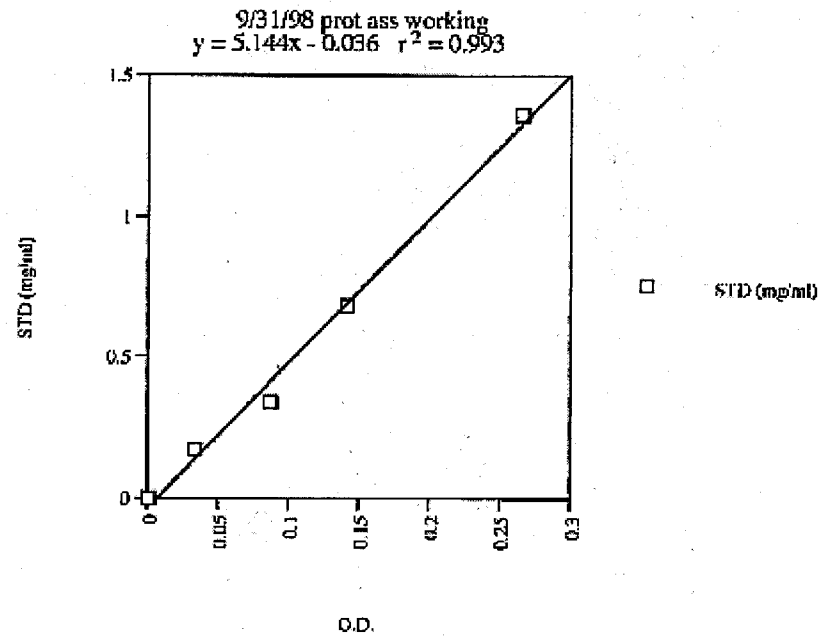
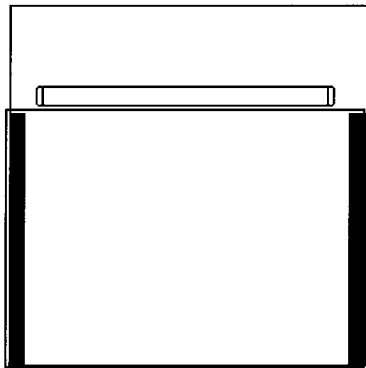
Figure 4 Bradford Protein Assay

Figure 5 NEpHGE



Tube Gel

**First Dimension NEpHGE
-separation of proteins
by pI**



**Second Dimension NEpHGE
-separation of proteins by
molecular weight**

Results

A vesicle preparation was performed from rat skeletal muscle as described in the materials and methods in order to isolate and analyze a membranous fraction. The protein composition of each of the subcellular fractions collected during the vesicle preparation was analyzed by comparing the unknown protein bands within the samples with known protein standards added during SDS-PAGE. Most of the fractions were rich in protein with multiple bands in each lane. Samples F, H, I and K show little or no protein banding in the SDS-PAGE Figure 6 due to the low concentration of proteins in these samples. The samples are as follows: F) is supernatant after centrifugation at 4,500 x g for 15 minutes, H) is supernatant after the addition of KCl solution and ATP and ultracentrifuged at 80,000 x g for 90 minutes, I) is the supernatant after another centrifugation at 80,000 x g for 60 minutes, and K) the final pellet in KCl solution. These results and descriptions of each sample can be seen in Figure 6.

Next, western blot analysis of each of the samples was performed using sera from a myasthenia gravis patient with rippling muscles. Immunoreactivity was detected between MG/RMD patient's sera and protein bands in three of the subcellular fractions obtained including a whole

muscle sample. The samples showing immunoreactivity are A, B, and C described below (see Figure 6). Each of the reactive samples have similar protein compositions consisting of major bands around the molecular weights 29, 45 and 66 kDa with multiple lighter bands throughout the entire range of the SDS-PAGE gel. These results can be seen in Figure 7. Lane 1 shows the molecular weight standards added during SDS-PAGE having molecular weights of 29, 45, 66, 97.5 and 116 kDa. The protein composition of the whole muscle sample (A) as determined by SDS-PAGE is shown in lane 2 and the immunoreactivity of that sample with sera from a MG/RMD patient is shown in lane 3. The MG/RMD sera reacted with two protein bands in this sample, one band having a molecular weight around 97 kDa and the another band with a molecular weight between 45 and 65 kDa. In sample B, the supernatant of the whole muscle sample after centrifugation at 3,300 x g for 30 minutes, two bands were also detected. One band with a molecular weight between 45 and 66 kDa, and a smaller band around 97 kDa as shown in lane 5. The protein composition of this fraction can be seen in lane 4. Sample C, the supernatant after another centrifugation at 3,500 x g for 45 minutes to pellet mitochondria, showed identical results as the western blot of sample A. The protein composition of sample C is shown in lane 6 and the western blot of this sample with MG/RMD sera is

shown in lane 7. It is unclear as to why the 97 kDa band is much fainter in sample B as compared to the other samples showing immunoreactivity at the same molecular weight.

In an attempt to further characterize the proteins in the vesicle preparation samples showing reactivity, non equilibrium pH gel electrophoresis or NEpHGE was performed. NEpHGE is a two dimensional form of gel electrophoresis which further separates proteins based on electrical charge and molecular weight as opposed to SDS-PAGE which separates proteins based on molecular weight alone. After six NEpHGE tube gels were run (2 for each sample showing immunoreactivity), one gel was stained with coomassie brilliant blue stain while the others were frozen for future western blotting. The coomassie stained gel showed an atypical staining pattern that indicated problems with the NEpHGE method used in the lab led to the distortion of the gel pattern. Normal results from a NEpHGE gel would separate proteins first by pH with the basic proteins on one end of the gel and the acidic proteins at the opposing end with intermediate pI (isoelectric point) in between. In the second portion of the procedure the proteins were then further separated by molecular weight, the high molecular weight proteins stay near the top of the gel with the intermediate sized proteins in the middle, and lower weight proteins at the

bottom of the gel. The gels that were stained after the second dimension with coomassie blue showed an inconsistent immuno-staining pattern. When western blotted with patient sera, the NEpHGE gels either showed no immunoreactivity or showed immunoreactivity patterns not usable for analysis (see Figure 8).

Overall, the results from the vesicle preparation did not indicate new information nor did the vesicle preparation appear to be a pure subcellular fraction. Results from a previous study indicated that there were at least two proteins at high (300-500 kDa) and intermediate (~200kDa) molecular weights that were not seen on our vesicle preparation gels or blots. For this reason we sought out a protocol that would allow us to isolate a T-tubular membrane in a higher concentration. The T-tubular region is of interest since this is the region of Ca^{++} entrance into the cell and antigens in this region may be responsible for rippling muscle symptoms. The T-tubular membrane proteins may have been present in the vesicle preparation fractions but possibly not in a large enough concentration to be detected during the western blot analysis.

A modified T-tubule isolation procedure from Florio *et al.* (1992) was used to isolate the T-tubular membranes. The final sample was western blotted with anti-DHPR as a primary antibody to determine if the fraction

contained T-tubular membrane. Since DHPR is associated only with the T-tubular membrane, detection of DHPR in a western blot showed that we did indeed collect T-tubular membrane.

Four total fractions were collected during the T-tubule isolation and were western blotted with various sera and antibodies. The four fractions include the following. Fraction 1 is the discarded material after the first centrifugation at 3600 rpm in a Beckman J6 rotor for 20 minutes. Fraction 2 is the pellet after centrifugation at 10,000 x g for 20 minutes. Fraction 3 is the supernatant after centrifugation at 75,000 x g for 90 minutes and fraction 4 is the final T-tubule sample. The patient sera used for western blot analysis included a MG/RMD patient, a patient with MG but without the rippling muscle component, a MG patient with a thymoma, and a normal, non-diseased human. Results can be seen in Table 2.

The western blot using anti-DHPR antibody shows DHPR present in a detectable concentration in the final sample only (see Figure 9), which indicates that there is T-tubular membrane present in the final fraction. The blots performed with the sera from a MG/RMD patient detected a great deal of immunoreactivity in several bands of each fraction. Many bands were also detected within the stacking gel indicating many of the proteins were too large to enter into the resolving gel. The final fraction was also blotted

for the presence of AchR, dystrophin, and RyR. There was no immunoreactivity detected from any of these three antibodies. The results of these western blots can be seen in Figure 10. The sera from a patient with MG but without the rippling muscle component displayed some banding but not to the extent found with the MG/RMD patient. The patient with MG and a thymoma showed no reactivity (just a bit of background noise) as did the sera from a normal individual. Results illustrated in Figure 11.

In order to further characterize which proteins were displaying reactivity with patient sera, another isolation procedure was performed to isolate both T-tubular and sarcoplasmic reticular membranes. Since the previously attempted isolation for T-tubular membrane may have included portions of SR membranes, some of the immunoreactivity detected during western blot analysis of the T-tubule final sample may have been from proteins within the SR membrane. This SR/T-tubule isolation allowed further isolation of these closely associated membranes in order to determine whether T-tubules and/or SR contain antigens to the MG/RMD patient sera. This isolation procedure was followed according to Sabbadini *et al.* (1983).

Fractions from all supernatants and pellets were collected during the isolation procedure and all 15 fractions were then analyzed by SDS-PAGE and western blot analysis. The description of each fraction is shown in

Table 3 and the protein composition of each fraction as determined by SDS-PAGE is shown in Figure 12. The antibodies used to test the fractions from this isolation procedure include; anti-DHPR, anti-RyR, anti-AchR, anti-dystrophin, and sera from a MG/RMD patient. RyR presence is shown in Figure 13. The results of these blots are shown in Table 4. All western blots showed antibody immunoreactivity to one or more of the fifteen samples except the dystrophin antibody. Anti-AchR displayed immunoreactivity with 3 of the fractions and was assumed to have been isolated and removed since it is not reactive with any of the samples taken from the remaining steps of the isolation procedure. Both the anti-DHPR and anti-RyR antibodies displayed immunoreactivity with the majority of the fractions. Both are absent from several fractions taken at the latter portion of the isolation procedure but do react with the final pellet. Since both DHPR and RyR are present in the final pellet, it is probable that a good separation of the two membranes was not achieved. The sera from the MG/RMD patient was extremely reactive with fractions taken from the first part of the isolation but was not reactive with any of the fractions taken from the last half of the procedure including the final pellet fraction which displayed reactivity to both anti-DHPR and anti-RyR antibodies.

One problem arose that impaired the ability to collect data during the last portion of this project. The swinging bucket rotor necessary for the T-tubule/SR isolation procedure, was unavailable for use due to mechanical problems. The ultracentrifugation that was to be performed in the swinging bucket rotor was instead performed using an anglehead rotor with unsatisfactory results. A complete separation of T-tubular membrane from SR membrane was not possible with the type of rotor used.

Figure 6 Gel of Vesicle Preparation Fractions

Lane A	Whole rat muscle sample
Lane B	Supernatant of muscle sample after centrifugation at 3,300 x g for 30 minutes.
Lane C & D	After centrifugation again at 3,500 x g for 45 minutes, samples were taken from both the supernatant (C) and the mitochondrial pellet (D).
Lane E	Supernatant after ultracentrifugation at 44,000 x g for 60 minutes.
Lane F & G	After centrifugation at 4,500 x g for 15 minutes samples were taken from the supernatant (F) and the pellet (G).
Lane H	Supernatant after addition of KCl solution and ATP and ultracentrifugation at 80,000 x g for 90 minutes.
Lane I	Supernatant after another ultracentrifugation at 80,000 x g for 60 minutes.
Lane J	Final pellet
Lane K	Final pellet in KCl solution

Figure 6 Gel of Vesicle Preparation Fractions

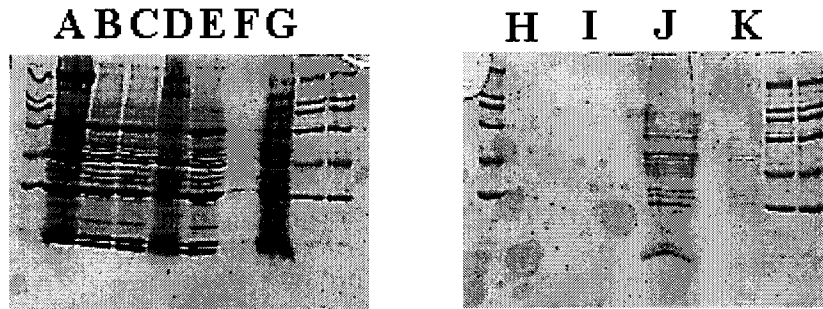


Figure 7 Immunoreactivity of Fractions with MG/RMD Sera

- Lane 1 Molecular weight standards
- Lane 2 SDS-PAGE of sample A
- Lane 3 Western blot of sample A showing immunoreactivity to sera
from a MG patient with the rippling muscle component.
- Lane 4 SDS-PAGE of sample B
- Lane 5 Western blot of sample B showing immunoreactivity to sera
from a MG patient with the rippling muscle component.
- Lane 6 SDS-PAGE of sample C
- Lane 7 Western blot of sample C showing immunoreactivity to sera
from a MG patient with the rippling muscle component.

Figure 7 Immunoreactivity of Fractions with MG/RMD Sera

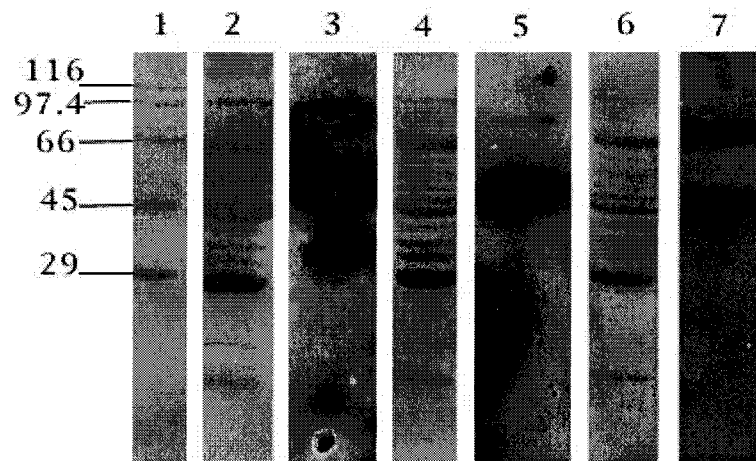


Figure 8 NEpHGE Results

This figure illustrates the inconsistent banding patterns obtained from the NEpHGE analysis. Picture A shows a NEpHGE coomassie stained gel. Pictures B and C both show the immunoreactive banding pattern obtained during western blot analysis.

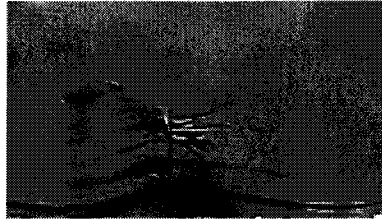
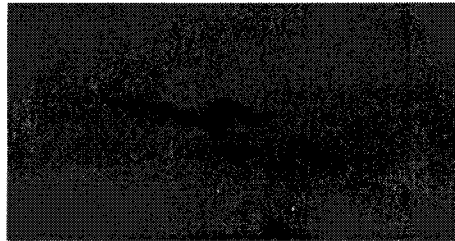
Figure 8 NEpHGE Results**A****B****C**

Table 2 T-tubule Western Blot Results

This figure illustrates the immunoreactivity of all the samples obtained from the T-tubule isolation procedure with commercial antibodies DHPR, RyR, AchR, and dystrophin. Sera from different patients including a MG/RMD patient with thymoma, a MG/RMD patient, a MG patient without RMD, a MG patient with thymoma, and a normal individual were also used. The T-tubule final fraction reacted with DHPR, one patient with MG, and the patient with MG/RMD with thymoma.

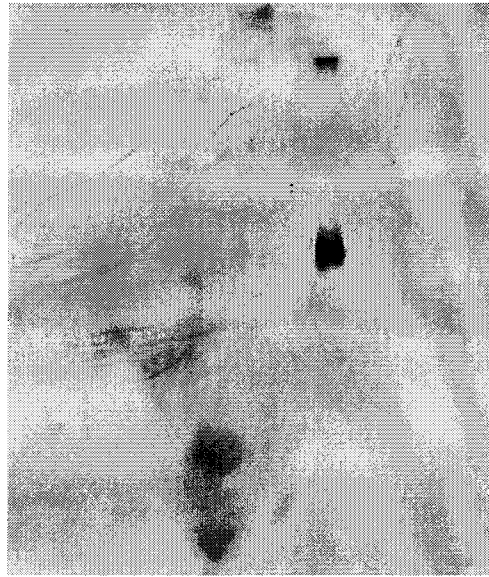
Table 2 T-tubule Western Blot Results

Antibodies/Patient Sera	<u>Fraction 1</u>	<u>Fraction 2</u>	<u>Fraction 3</u>	<u>Fraction 4</u>
DHPR	-	-	-	+
MG/RMD/ thymoma	++	++	++	++
MG alone	-	-	-	+/--
MG/RMD no thymoma	-	-	-	-
MG/ thymoma	-	-	-	-
Normal Individual	-	-	-	-
RyR	No data	No data	No data	-
AchR	No data	No data	No data	-
Dystrophin	No data	No data	No data	-

Figure 9 Presence of DHPR in T-tubule Final Fraction

The presence of DHPR was found in the final T-tubule sample (4) but is absent in the fractions 1, 2, and 3. Fraction 1 is the discarded material after the first centrifugation at 3,600 rpm in a Beckman J6 rotor for 20 minutes. Fraction 2 is the pellet after the next centrifugation at 10,000 x g for 20 minutes and fraction 3 is the supernatant after centrifugation at 75,000 x g for 90 minutes.

Figure 9 Presence of DHPR in T-tubule Final Fraction

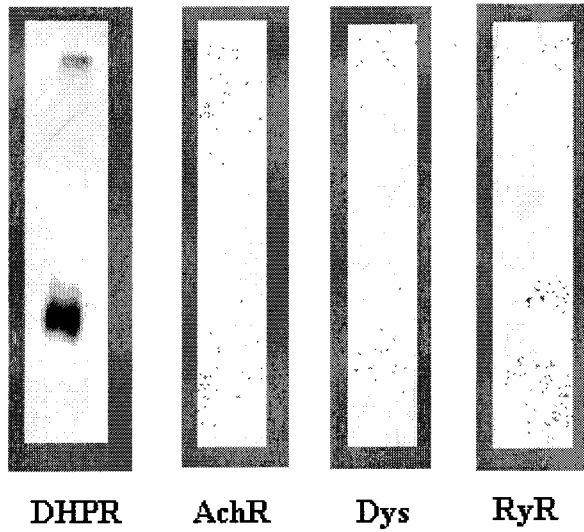


Fractions: 1 2 3 4

**Figure 10 T-tubule Final Fraction Immunoreactivity with
Commercial Antibodies**

The final T-tubule fraction was analyzed by western blot to determine the purity of the sample and the effectiveness of the isolation procedure. DHPR was detected in the sample (lane 1) while AchR, dystrophin, and RyR (lanes 2, 3, and 4 respectively) did not display any immunoreactivity with the final T-tubule sample.

**Figure 10 T-tubule Final Fraction Immunoreactivity with
Commercial Antibodies**



**Figure 11 Immunoreactivity of T-tubular Fraction with
Patient Sera**

All sera was tested against the final T-tubule sample. Lanes A B and C are all patients with MG. Lane D is MG/T. Lane E is MG/RMD. Lane F is MG/RMD/T. Only lanes C and F show immunoreactivity which is not expected since lane C is MG only---no RMD. (May be an extreme case with a thymoma undiagnosed or may be experiencing the beginnings of RMD.)

Figure 11 Immunoreactivity of T-tubular Fraction with Patient Sera

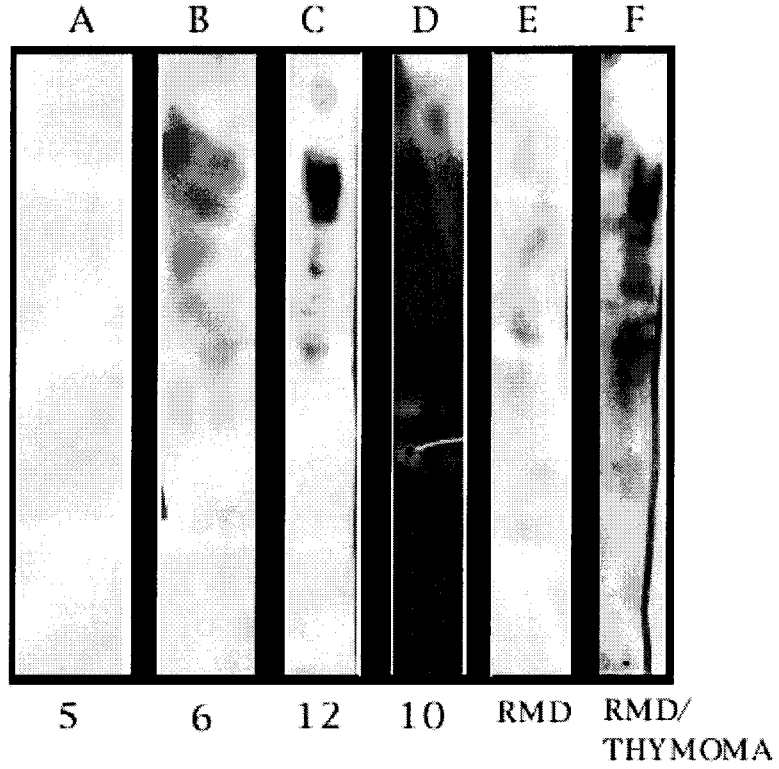


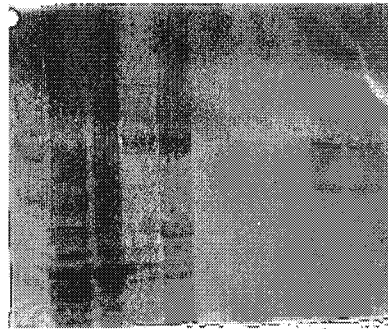
Table 3 Description of T-tub/SR Isolation Fractions

<u>Fractions:</u>	<u>Fraction Descriptions:</u>
1	Whole muscle after homogenized in a Waring blender
2	Pellet after centrifugation at 15,000 x g for 20 minutes
3	Supernatant after centrifugation at 15,000 x g for 20 minutes
4	Fraction 3 after filtered through gauze
5	Pellet after centrifugation at 40,000 x g for 90 minutes
6	Supernatant after centrifugation at 40,000 x g for 90 minutes
7	Pellet after centrifugation at 15,000 x g for 20 minutes
8	Supernatant after centrifugation at 15,000 x g for 20 minutes
9	Supernatant after centrifugation at 40,000 x g for 90 minutes
10	Loaded sample before sucrose gradient
11	T-tubule sample after first sucrose gradient centrifugation
12	T-tubule final sample from tube #1
13	T-tubule final sample from tube #2
14	Final SR top layer
15	Final SR pellet

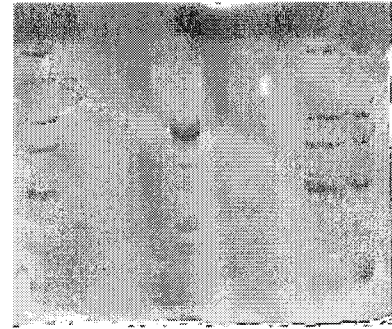
Figure 12 Protein Composition of SR/T-tubule Fractions

This figure shows the protein composition of the 15 fractions from the SR/T-tubule isolation procedure. The S lanes are the protein standards used during SDS-PAGE. The numbered lanes correlate with the descriptions of the fractions listed in table 3.

Figure 12 Protein Composition of SR/T-tubule Fractions



S 1 2 5 7 9 10 11 S S

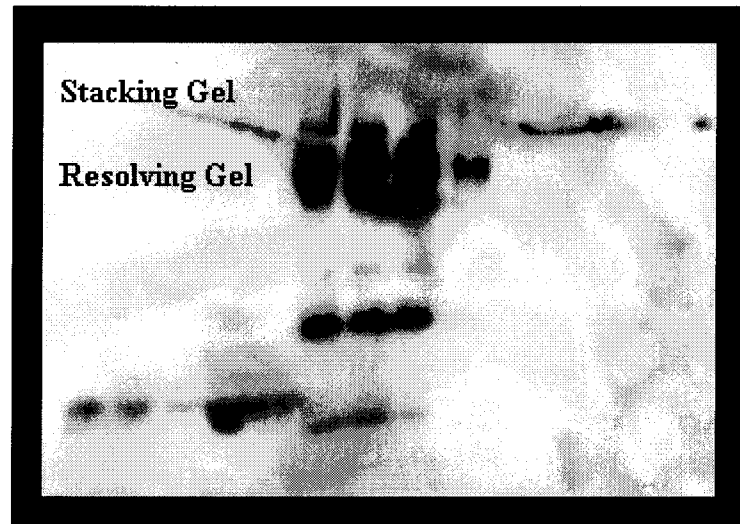


S 12 13 14 15 S S

Figure 13 Presence of RyR in SR/T-tubule Fractions

The presence of RyR in the fractions of the SR/T-tubule isolation can be seen in this figure. Immunoreactivity can be seen in both the stacking and resolving gels. The immunoreactivity detected at the bottom of the resolving gel may be the result of RyR breakdown.

Figure 13 Presence of RyR in SR/T-tubule Fractions



**Table 4 Immunoreactivity of SR/T-tubule Fractions with
Different Antibodies and Patients' Sera**

Fractions:	DHPR	RyR	AchR	Dystrophin	MG/RMD
1	+	+	-	-	+
2	-	+	-	-	+
3	+	+	+	-	+
4	+	+	+	-	+
5	+	+	+	-	+
6	+	+	-	-	+
7	+	+	-	-	+
8	+	+	-	-	-
9	+	-	-	-	-
10	+	-	-	-	-
11	-	-	-	-	-
12	-	-	-	-	-
13	-	-	-	-	-
14	-	-	-	-	-
15	+	+	-	-	-

Discussion

The goal of this study was to further characterize autoantibodies found in MG/RMD patients' sera through analysis of subcellular fractions of skeletal muscle. One hypothesis of this study was that MG/RMD patients have antibodies to proteins in the sarcoplasmic reticular and T-tubular portions of the skeletal muscle cell. It is already well established that MG patients have a great deal of autoantibodies due to their condition, however autoantibodies associated with MG/RMD patients have not yet been thoroughly investigated. Autoantibodies present in MG patients include those against nicotinic acetylcholine receptors (290 kDa), thyroid, and skeletal muscle proteins including titin, actin, myosin, tropomyosin, alpha-actinin and portions of ryanodine receptor (RyR) (300-400 kDa) (Aarli, 1990; Gautel, 1993; Penn, 1986; Ohta, 1990; Pagala, 1990; Williams, 1986).

In a previous study performed by Thomas Watkins M.S., sera of a MG/RMD patient was found to have immunoreactivity with whole muscle proteins when analyzed using western blot techniques. Immunoreactivity to a high molecular weight protein (~400 kDa) was found as was reactivity to a molecular weight protein approximately 200 kDa in size (an intermediate molecular weight protein) and a relatively low molecular weight protein (~77 kDa in size). This reactivity was also found in sera from other

MG/RMD patients. MG patients without the rippling muscle component did display some reactivity around 40-70 kDa which can probably be explained as reactivity to acetylcholine receptor (AChR) subunits. These subunits range in molecular weight from 39 to 64 kDa (Watkins, 1999).

Sarcoplasmic reticulum and T-tubular regions of the skeletal muscle cell were targeted in this study because Ca^{++} enters into the cell at this location via ryanodine receptor Ca^{++} channels. These have a molecular weight of between 300 and 400 kDa and may explain the immunoreactivity found in this range. Immunocytochemistry performed during the previous study displaying immunoreactivity in a banding pattern in the T-tubular region of a myocyte suggests this area may contain autoantigens to MG/RMD patient sera. See Figure 3. This location also correlates to the possible presence of mechanosensitive channels (MSC's) or channels activated or inactivated by stretch of the muscle cell since Ca^{++} enters the cell in this region. A conformational change of MCS's may occur due to the presence of autoantibodies found in MG/RMD patients and may account for the rippling muscle component by simply allowing these channels to leak Ca^{++} into the myocyte.

The first step taken in this study was the preparation of vesicular membranes from the myocytes according to de Meis *et al.* (1971). Fractions

were collected at all stages of the vesicle preparation and each fraction was then analyzed by western blot techniques. The final fraction did not show any reactivity to the MG/RMD sera. This may be because the proteins of interest were not isolated in the final fraction in a significant concentration to react with the autoantibodies of the sera. Other than the whole muscle homogenate sample (A), only two of the fractions, samples B and C, reacted with the patient sera. Fraction B was the supernatant after the whole muscle homogenate was centrifuged at 3,300 x g for 30 minutes. Fraction C was the supernatant after another centrifugation at 3,500 x g for 45 minutes.

Descriptions and results are shown in Figures 6 and 7. Immunoreactivity was detected in all three samples at ~97 kDa and again between 45 and 66 kDa. These bands could possibly be acetylcholine receptor subunits.

However, the intermediate and high molecular weight proteins reactive in the previous study were not seen in this isolation procedure therefore another isolation procedure, specific for T-tubular membrane, was performed.

T-tubular membranes were isolated according to a modified procedure from Florio *et al.* (1992). Four fractions were collected over the course of the isolation procedure, including the final T-tubular sample, and tested by SDS-PAGE and western blot analysis techniques. Table 2 shows the immunoreactivity detected during western blots of the fractions with several

different antibodies and patients' sera. Fraction 4 is of main interest since it should contain the isolated T-tubular membranes. This fraction was analyzed by western blot to determine the contents. The final fraction contains DHPR. Since DHPR is a T-tubule associated channel protein, its reactivity with the final sample indicates that T-tubule membrane is present in the sample. DHPR was not detected in any of the other three fractions possibly because it is not present in a large enough concentration. The final sample was also blotted against AchR, dystrophin, and RyR. No immunoreactivity was detected. This indicates that there is little or no contamination from plasma membrane associated with AchR which is present at the neuromuscular junction. This also indicates that the sample may be isolated T-tubular membrane since RyR is not present. RyR is a channel protein associated with the sarcoplasmic reticulum. Since the final sample showed no reactivity with RyR it may be assumed that sarcoplasmic reticular membrane is not present in the sample. The T-tubular membrane and SR are normally closely associated, and therefore the absence of SR in the final fraction may be indicative of a fairly pure T-tubular fraction.

The T-tubule fraction did react with sera from two patients: 1) a MG/RMD/T patient and 2) a patient with MG only. Although sera from other types of patients (MG/T, MG/RMD, and a normal healthy individual)

were tested, there was no immunoreactivity detected. This could possibly illustrate that the antigens reactive in the final fraction respond to antibodies found in some MG patients without the RMD component but is not found in all MG patients. Banding patterns were in the very high and high molecular weight ranges which may be consistent with the results obtained from a previous study (Watkins, 1999).

In order to further characterize autoantigens, another isolation procedure was performed to isolate both T-tubular and sarcoplasmic reticular membranes. This isolation procedure was followed according to Sabbadini *et al.* (1983). In the SR/T-tubular membrane isolation, 15 fractions were collected and examined by SDS-PAGE and western blot analysis. Fraction descriptions are shown in table 3. Western blot results are shown in table 4. All were blotted with MG/RMD sera and four commercial antibodies against DHPR, AchR, RyR, and dystrophin. Dystrophin did not appear in any of the 15 fractions. Thus far, the lab has not received a positive blot with the dystrophin antibody indicating that there may be a problem with the antibody. A possible reason may be that we were using the wrong form of the antibody (it may not be specific for the type of muscle contained in the samples). AchR was present in three of the 15 fractions. It can probably be assumed that the AchR was removed from the

isolation in the pellet referred to as fraction 5 since this pellet is normally discarded and is the last fraction displaying reactivity with the AchR antibody. DHPR and RyR are both highly reactive to the fractions from the first portion of the isolation procedure but are undetected in most of the last fractions except the final pellet. DHPR is present in all samples (except a pellet removed and discarded after the first centrifugation) before the sucrose gradient portion of the isolation. RyR is also present in all fractions before a final centrifugation preceding the sucrose gradient portion of the procedure. However, both DHPR and RyR are detected in the final pellet. The final pellet should be isolated SR that had been separated from T-tubular membranes. A good separation was unfortunately not achieved since there is T-tubule associated DHPR present in the fraction.

The sera from the MG/RMD patient was reactive to fractions 1 through 7 but did not react with any fractions from the remainder of the isolation procedure including the final pellet containing SR and T-tubular membrane. The MG/RMD patient sera should have shown reactivity with the final pellet since the T-tubular membrane containing final fraction of the previous T-tubule isolation procedure showed immunoreactivity to the sera. Since the final fractions of both isolation procedures contained T-tubules, the MG/RMD sera should display immunoreactivity with both fractions.

Recent work performed in the lab has yielded a positive immunoreactivity detection between the final fraction of the SR/T-tubule isolation and the MG/RMD sera. The recent work performed has not however been able to achieve a good separation between the SR and T-tubular membrane. DHPR and RyR are again both present in the final pellet which should be SR only and therefore not be reactive to DHPR.

Inconsistent immunoreactivity results from the data described may be due to the unavailability of a necessary rotor required for the SR/ T-tubule isolation. The sucrose gradient portion of the isolation was performed with an anglehead rotor instead of a swinging-bucket rotor normally utilized for this type of procedure. The recent work mentioned above was performed on the correct type of rotor. This corrected the problem of negative immunoreactivity between the final sample and MG/RMD sera but still did not permit an adequate separation of the SR and T-tubular membranes as expected.

To conclude, three isolation procedures were performed. A vesicle preparation to isolate vesicle membranes from myocytes, a T-tubular isolation to isolate T-tubular membranes, and an SR/T-tubule isolation for the purpose of collecting isolated sarcoplasmic reticular and T-tubular membranes. The vesicle preparation provided no new data. The T-tubular

isolation procedure illustrated that the final T-tubule containing pellet displayed immunoreactivity with some MG sera and with sera from some MG/RMD patients. Therefore, T-tubule antibodies may be manufactured by some MG patients but do not appear to be produced by only those MG patients with a rippling muscle component. The SR/T-tubular isolation final fraction does not show immunoreactivity with MG/RMD sera suggesting that the MG/RMD patients have no antibodies to SR membrane proteins. Continuing work in the lab may indicate otherwise (perhaps a different isolation procedure is required). In this continuing work, the reactivity between the MG/RMD sera and the SR/T-tubule containing final pellet is located in the same molecular weight region as reactivity displayed by DHPR and RyR antibodies suggesting a possibility that MG/RMD patients may have antibodies to SR and T-tubular proteins.

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INTEROFFICE MEMORANDUM

TO: DR. GARY WALKER
FROM: CHERYL COY
SUBJECT: LACUC PROTOCOL MODIFICATIONS
DATE: 06/03/99
CC: LACUC FILE

Dr. Stephen Flora, IACUC Chair has reviewed the modifications you submitted to your protocol #01-99 and determined they met the conditions outlined by the IACUC committee with the exception of the signature of your co-investigator on the declaration page. I have enclosed that page for Thomas Watkins to sign. Please have him sign and return it to me and then you will have met all conditions.

Thank you.



