Characterization of Skeletal Muscle Antibodies in Patients with Autoimmune Rippling Muscles and Myasthenia Gravis

by

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I. Abstract

Rippling muscle disease has been previously described by Torbergsen, as a rare autosomal dominant condition characterized by: muscle stiffness after resting, localized mounding of muscle after resting and electrically silent wave like contractions after stretching (i.e. rippling muscles). In 1990 Ansevin described a patient displaying electrically silent rippling muscles with no evidence of Myasthenia gravis. In 1995 the patient returned for treatment for myasthenia gravis (MG). At the time of treatment for MG the rippling muscles were absent. It was theorized that the rippling muscles, in this patient, were the result of an autoimmune condition related to the one that brought on the MG. We have examined serum obtained from this patient with active rippling symptoms and when the rippling symptoms had abated, by western blotting. We have also examined serum obtained from two other patients with rippling muscles and MG. Results indicate that high and intermediate molecular weight antigens in skeletal muscle are recognized by autoantibodies in patients with rippling muscles, which are not present in patients with myasthenia gravis alone. The data suggest autoantibodies may be binding to stretch activated channel proteins causing the symptoms of rippling muscle.

Acknowledgments

I wish to thank my advisor Dr. Gary Walker, for his patience and gift of friendship. My appreciation to Carl Ansevin, M.D. for his guidance, and special gift of encouragement. These mentors have indelibly etched a place not only in my professional life but my personal life as well. To Dr. Paul Petersen and Dr. Mark Womble my special thanks for serving on my committee and for input and aid on this project. My colleagues Stacy Raab and Richard Kandrac whose assistance and camaraderie helped lighten the hours in the lab. For his help and love I thank my father. My mother's unconditional love and belief in me illuminate the cloudy moments and make the sometimes bumpy road of life a little less rocky. To my grandparents, the Beach's, love and thanks for being there.

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II. Introduction

Rippling muscle disease was first characterized by Torbergsen in 1975 while examining a family with a disease resembling myotonia congenita. Rippling muscles as observed by Torbergsen were different from myotonia congenita in some distinct ways. Since Torbergsen a number of authors have gone on to report similar cases of rippling muscle disorders with the following common symptoms: muscle stiffness after resting, a local mounding of muscle after percussion, and wave-like rippling of muscle after stretching or percussion, that appears electrically silent (Ricker et al., 1989; Kosmorsky et al., 1995 Ansevin et al., 1996). Rippling muscle disease is a rare autosomal dominant (Ricker et al., 1989) condition that has been described in the United States, Scandinavia, and Germany. Further studies have been able to link the gene responsible for the disorder to the long arm of chromosome one, although a later study of two separate families with the disorder did not show the genetic defect at the same locus. These results are evidence that a similar phenotype of this disorder may be the result of genetic heterogenicity (Stephan et al., 1994).

The cases of rippling muscles described previously were the result of a genetic disorder in otherwise healthy patients (Torbergsen et al., 1975; Ricker et al., 1989; Burns et al., 1994). Our studies have focused initially on a

patient described by Ansevin et al., in 1996. The patient exhibited rippling muscles with myasthenia gravis (MG) and a thymoma. This is the first case described of rippling muscles and MG associated with a thymoma that has been described. This patient sought treatment at the age of 56 in 1990. At that time the patient was exhibiting classical symptoms of rippling muscles, stretch and percussion activated wave like contractions in the arms and legs accompanied by weakness in the aforementioned areas. Importantly, the ripples in the muscle were also shown to be electrically silent, as in the previously described cases. The patient had nine siblings with no neuromuscular disorders and a family history also free of neuromuscular disorder. At the time of initial diagnosis there was no evidence of myasthenia gravis. The patient was not seen again until 1995 when the diagnosis of MG was made in correlation with severe muscle weakness, fatigability and elevated titers of anti-acetylcholine receptor, and other skeletal muscle antibodies. The patient was treated with pyridostigmine and prednisone improving his condition. The patient has also undergone two plasmaphereses and a thymoma removal. Symptoms of the MG improved and the rippling muscles ceased. The preceding clinical evidence led Dr. Ansevin to assert that the patient's rippling muscles may have been precipitated by an autoimmune response similar to that which brought on his

MG (Ansevin et al., 1996). Other cases of rippling muscle disease have since been reported.

The link between rippling muscles and myasthenia gravis with a thymoma necessitates a further discussion of these autoimmune conditions and their relation to this study.

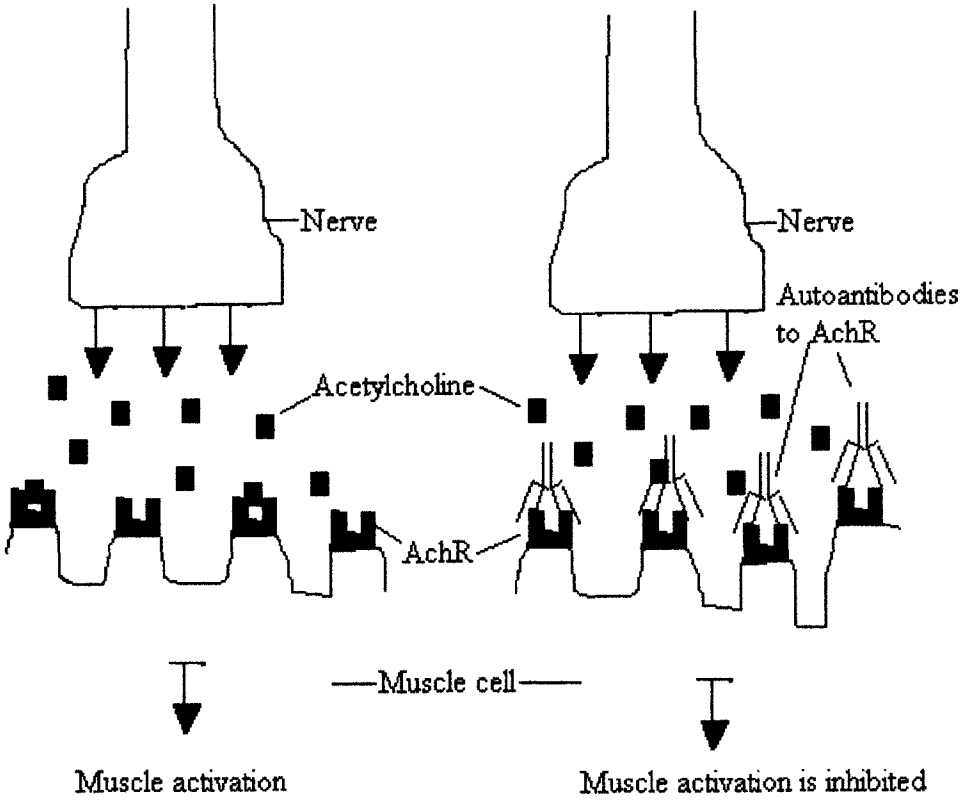
Autoimmune diseases are divided into two classes: organ specific and systemic autoimmune disease. Systemic autoimmune diseases result from a generalized defect in the regulation of immune cells resulting in hyperactive T and B cells. Tissue damage from this state is widespread from cell mediated responses and from cellular damage caused by autoantibodies or the accumulation of immune complexes. Organ specific diseases are so called because they are characterized by only one type of organ or tissue experiencing damage from the immune system by either humoral or cell mediated responses. The function of a target organ may be blocked or stimulated by antibodies (Kuby et al., 1994). Myasthenia gravis is a well-characterized autoimmune disease of the organ specific type. In this disorder, the muscles of the face and throat are most commonly affected. However, many other muscles may also be involved. Affected muscles fail to receive signals from the nerves, due to a lack of functional acetylcholine receptors. This abnormal muscle transmission is associated with circulating

autoantibodies that bind to acetylcholine receptors (AchR) of skeletal muscle inhibiting their stimulation by acetylcholine (Penn et al., 1986; Kuby et al. 94). This loss of signal to the muscles is responsible for the symptoms of drooping and weakness. The blocking action of antibodies on acetylcholine receptors is summarized in figure 1.

Current research areas in myasthenia gravis have focused on anti-muscle antibodies produced in patients with MG and thymoma. These patients account for around fifteen percent of MG patients. Many of these patients exhibit autoantibodies to various proteins of striated muscle, such as titin, myosin, actin, and alpha-actinin (Skeie et al., 1996). Early studies in this area have clearly shown that myasthenia gravis patients' sera possesses autoantibodies to skeletal muscle A-bands and I-bands (Strauss et al., 1967; Vetters et al., 1967). Studies by Ohta et. al 1989 showed that there is a very high incidence of autoantibodies of the IgG class to myosin and actin. The same studies also showed that in the patients with MG and a thymoma there were also autoantibodies to tropomyosin and alpha-actinin in significantly higher titers than in control and non-thymoma patients.

About 50% of thymoma MG patients have been shown to exhibit IgG autoantibodies to a sarcoplasmic reticulum protein with a molecular weight around 320 kDa (Mygland et al., 1991). The only SR protein with similar

Figure #1


Nerve
Nerve
Autoantibodies
to AchR
Acetylcholine
AchR
Muscle cell
Muscle activation
Muscle activation is inhibited

electrophoretic characteristics is the calcium release channel protein called ryanodine receptor (RyR). Recent reports have placed the weight of the calcium release channel protein at 400 kDa (Lai et al., 1990). Several other reports however have placed the weight of this protein at a range of 300-400 kDa (Zorzato et al., 1986). The main cellular activity of ryanodine receptor is excitation-contraction coupling, leading to the increase in sarcoplasmic reticulum Ca^{2+} permeability triggered by cell depolarization. Ryanodine receptors are also believed to be a superfamily of intracellular Ca^{2+} release channel proteins that may be found in other tissues that do not undergo excitation-contraction coupling, such as neurons and epithelial cells (Coronado et al., 1994). Ryanodine receptors also respond to the second messenger, cyclic adenosine diphosphate ribose (cADPR) and release Ca^{2+} (Takasawa et al., 1993).

Protein-protein interactions may also trigger a conformational change in the ryanodine receptor that causes opening of the channel leading to an increase in myoplasmic Ca^{2+}. There are a several non-plasma membrane proteins that may play a role in the Ca^{2+} gating of the ryanodine receptors. These proteins include: calmodulin, triadin, a 60 kDa protein, a 106 kDa protein, and FK506 binding protein. The interactions of these proteins with

the ryanodine receptor may be of importance in cell-signaling cascades (Corronado et al., 1994).

It is important to note that there is a correlation of anti-ryanodine receptor antibodies to the severity of disease in myasthenia gravis patients. A study by Mygland et al 1994, suggests that the presence of ryanodine receptor antibodies and a severe form of thymoma associated myasthenia gravis have a strong relationship between disability and peak serum levels of ryanodine receptor (Mygland et al., 1994). A study by Skeie et. al., 1996 has made several assertions as to the role of ryanodine receptor in autoimmune human disease. The study reports that there have been reports of defective excitation-contraction coupling in patients with MG and a thymoma (Nielsen et al., 1982; Skeie et al., 1996). In addition, other authors (Emlen et al., 1992; Morgan et al., 1992) suggest that antibodies directed to intracellular antigens like ryanodine receptor can pass the cell membrane and have functional effects. This evidence suggests that ryanodine receptor autoantibodies may have a pathogenic role of their own, in addition to the role of anti-AchR, in MG patients.

Further suggestions for the appearance of these autoantibodies in MG thymoma patients is provided by the studies of Takeshima et al., 1989. They show that there is some sequence homology between the amino acid residues

4628-4861 of the ryanodine receptor and the residue 231-301 of the acetylcholine receptor alpha subunit which are recognized by autoantibodies in some MG patients. These epitopes shared by the RyR and AchR may possibly play a role in the initial sensitization of the autoreactive T helper cells in the thymoma (Skeie et al., 1996)

It has also been reported that patients with myasthenia gravis and a thymoma have antibodies to titin. Titin was first characterized by Wang et al., 1982, and was found to have a molecular weight of approximately 3000 kDa. Titin is a giant protein that provides most of the elasticity to relaxed striated muscle by connecting myosin to the Z-band. Titin is primarily a string of about 300 immunoglobulin domains and fibronectin type III domains. There is also a specific amino acid sequence (PEVK) found in the middle of the I-band segment. It is the unfolding and straightening of this domain, as well as the partially collapsed immunoglobulin segments that could account for the passive elasticity of skeletal muscle (Erickson et al., 1997). A study by Aarli et. al., 1990, suggests that MG thymoma patients have antibodies that react with a large muscle polypeptide that has the same electrophoretic mobility and the same immunoelectron microscopic localization as titin. Titin is present in both heart and skeletal muscle which correlates with the finding that MG thymoma patients have more cardiac

abnormalities than non thymoma MG patients, although the role of titin autoantibodies in cardiac muscle dysfunction is not clear (Aarli et al., 1990). In a later study of the MG thymoma patients who showed immunoreactivity to titin, all patients exhibited autoantibodies to the same 30 kD sequence of the titin molecule. This suggests a homogeneity among MG thymoma patients in regard to titin epitopes (Gautel et al., 1993).

A brief discussion regarding the role of muscle proteins and the microscopic anatomy of muscle tissue will yield a greater understanding of muscle disease. The microscopic anatomy of skeletal muscle consists of thousands of very long cylindrical muscle cell fibers. These fibers are covered by a sarcolemma, the plasma membrane of a muscle cell. The sarcoplasm is then filled with cylindrical arrays of parallel myofibrils extending lengthwise within the muscle fiber. The pattern of alternating light and dark bands within the myofibril give the muscle cell the appearance of being striated. Myofibrils are the contractile elements of muscle tissue and are composed of functional units called sarcomeres, which consist of three different types of filaments, thin filaments, thick filaments, and the elastic filament (Tortora et. al. 1993). There are narrow plate shaped regions called Z-discs that separate adjacent sarcomeres. Between the Z-discs, there is a darker A-band area that is indicative of the thick filament region. Within the

A-band, portions of the thin flaments overlap the thick filaments. There is also an I-band that contains the remainder of the thin filament length and is devoid of thick filaments. Please see figure 2 for a diagramatic representation of the sarcomere. The Z-disc passes through the center of the I-band. The alternation of the dark A-bands and the light I-bands is what gives the muscle fiber its striated appearance. There is a narrow H-zone in the center of each A-band. The H-zone is divided by the M line which is formed by protein molecules that connect adjacent thick filaments of opposite polarity.

The thick filaments are formed by myosin molecules which are shaped like two hockey sticks with their tails twisted together. Their heads are oriented outward. About 200 myosin molecules form each thick filament. The tails of the myosin molecules point toward the center of the sarcomere and the heads project outward to bind exposed actin molecules of the thin filaments.

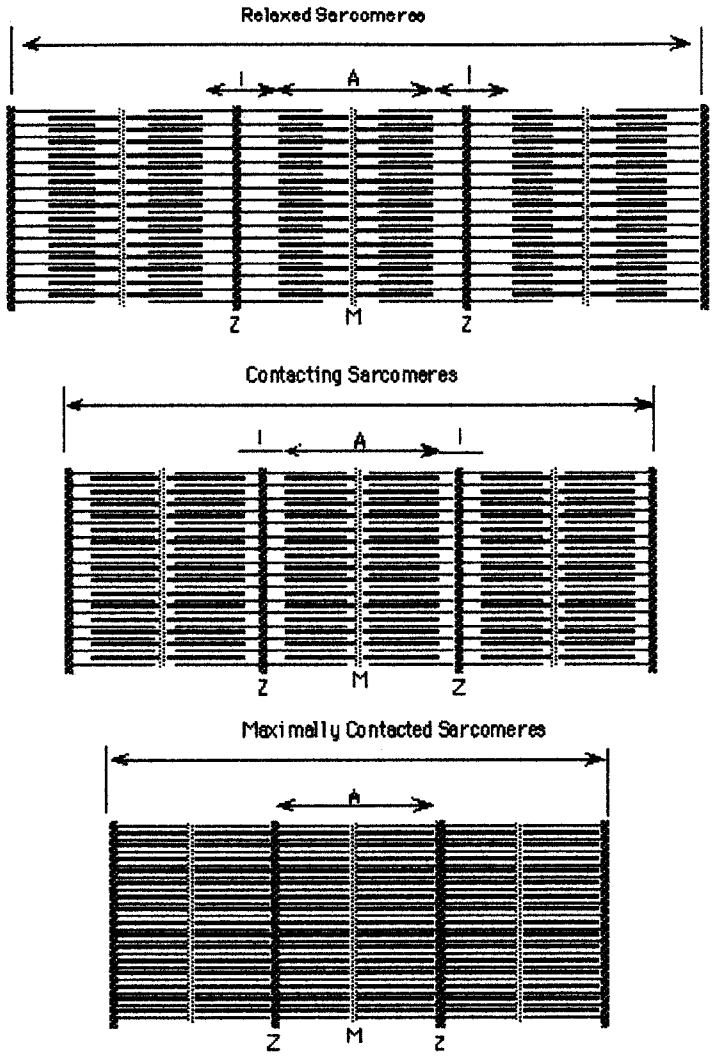
The thin filaments extend from anchoring points in the Z-discs and are primarily composed of the protein actin. Thin filaments also contain the actin binding proteins tropomyosin and troponin. Thin filaments are formed from 2 polymers joined together into a double helical structure. On each actin molecule is a myosin binding site which in resting skeletal muscle, is

covered by troponin/tropomyosin complexes, blocking the formation of myosin cross bridges.

The elastic filaments are made up of the protein titin. Titin anchors the thick filaments to the Z discs, thus stabilizing thick filament positioning. Titin also plays a role in the recovery of the resting sarcomere length when the muscle is stretched or contracted, thereby maintaining the elasticity of the muscle.

Figure 2 illustrates the contraction of sarcomeres. Note the shortening of the total length of the sarcomere and the movement of the thin filaments in relation to the thick filaments. The figure also shows the placement of the A-bands, the I-bands and the Z-discs (Tortora et. al., 1993).

The events of contraction and relaxation in skeletal muscle have been well characterized. First, nerve impulses arrive at the axon terminal end of a motor neuron and induce the release of acetylcholine (Ach) into the synaptic cleft Ach then diffuses across the cleft where it binds to an acetylcholine receptor protein located in the cell membrane of a skeletal muscle cell. Acetylcholine receptor (AchR) is the best characterized example of a neurotransmitter-gated ion channel. AchR has a total molecular weight of about 290 kDa It is composed of a ring of five membrane spanning subunits: two α subunits, and single β, γ, and, δ subunits (Noda et. al.


Relaxed Sarcomeres
I
A
I
Z
M
Z
Contacting Sarcomeres
I
A
I
Z
M
Z
Maximally Contacted Sarcomeres
A
Z
M
Z

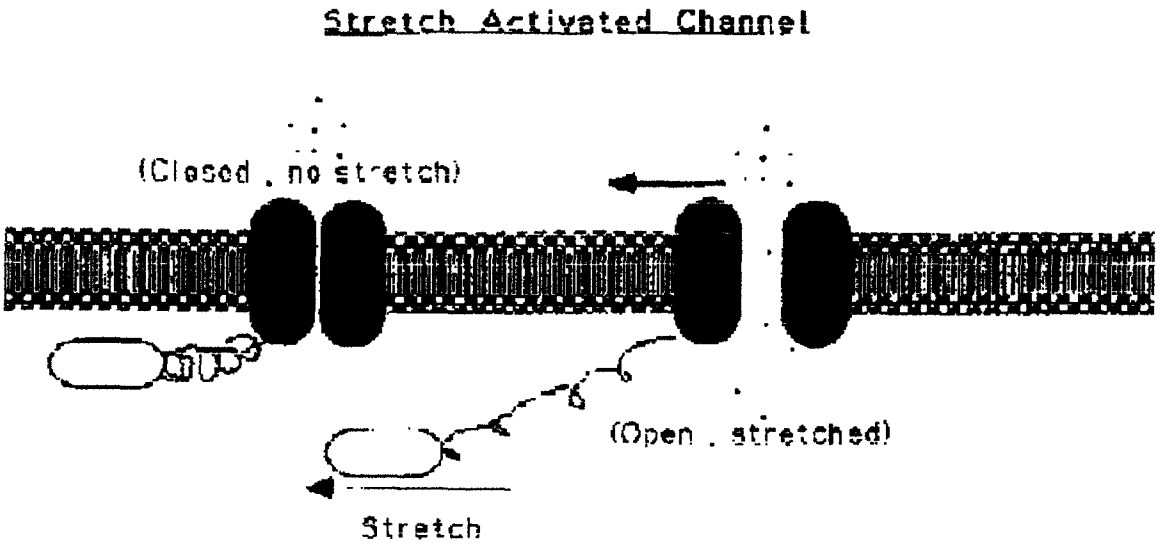
1983). The subunits have the following molecular weights: $\alpha \cong 39$ kDa, $\beta \cong$ 48 kDa, $\gamma \cong 58$ kDa and $\delta \cong 64$ kDa (Changeux et. al. 1984). Only the α subunit is capable of binding acetylcholine. The channel formed by these subunits opens transiently following the binding of 2 Ach molecules. It is the function of the AchR to rapidly convert a chemical signal from one cell to an electrical signal that is propagated along the next (Unwin et. al. 1993) Therefore the acetylcholine diffuses across the synaptic cleft, where the binding of two molecules of Ach cause a conformational change in the receptor allowing sodium ions to enter the cell, leading to a propagation of the action potential. Acetylcholine esterase then destroys acetylcholine, thus removing Ach until the next nerve impulse arrives and more Ach is released. Depolarization of the muscle sarcolemma results in the opening of calcium channels in the sarcoplasmic reticulum, and the release of Ca^{2+} ions into the sarcoplasm. Free calcium ions then bind to troponin on the thin filament, causing a conformational change in the tropomyosin and exposure of the myosin binding site on the actin molecules. These events result in activation of the myosin ATPase cycle and lead to muscle contraction. Myosin thick filaments pull the opposing thin filaments toward the center of the sarcomere, resulting in sarcomere shortening. The calcium release channels on the sarcoplasmic reticulum close when the membrane potential returns to the

resting potential. ATP powered calcium pumps lower the level of Ca^{2+} in the sarcoplasm by active transport of calcium back into the sarcoplasmic reticulum. The troponin-tropomyosin complex then slides back into position blocking the myosin binding sites on actin, and the muscle relaxes (Tortora et. al. 1993).

It is well established that myasthenia gravis patients with a thymoma have a wide spectrum of autoantibodies to various proteins in skeletal muscle (Strauss et al., 1967; Vetters, 1967; Nielsen et al., 1982 Zorzato et al., 1986; Ohta et al., 1990; Mygland et al., 1991; Mygland et al., 1994; Skeie et al., 1996). It has also been asserted that rippling muscle disease may also be caused by an autoimmune condition produced by these muscle autoantibodies (Ansevin et al., 1996). Rippling muscle disease characteristics also suggest that this condition may be caused by autoantibodies to mechanosensitive ion channels. Mechanosensitive ion channels were first described by Guchray in chick skeletal muscle (Guchray et al., 1984). These can be either stretch activated (SACs) or stretch inactivated (SICs) channels. They have been described in skeletal, cardiac, smooth muscle and other tissues (Guharay et al., 1985; Sackin, 1995; Kirber et al 1988; Naruse et al., 1993). Mechanosensitive channels may relate to rippling muscles since it has been observed that rippling muscles are stretch and percussion activated, not

triggered by neural sources, and the ripples are electrically silent (show an action potential independent mechanism) It is currently unknown how the rippling travels from myofiber to myofiber. Ricker et al. (1989) found the speed of the rippling to be about 0.6 meters per second, which is only about one tenth of the conduction speed of a muscle fiber action potential. These observations are consistent with the rippling phenomena being propagated by stretching of the sarcolemma. Stretching the muscle may open SACs, thereby allowing cations and small numbers of Ca^{2+} ions into the myofiber. The small amount of inflowing Ca^{2+} may cause contraction of the muscle fiber in the manner described above. This contraction may then mechanically disturb (i.e. stretch) adjacent myofibers and cause the ripple to spread without electrical activity.

The action of mechanosensitive calcium channels are illustrated in figure 3. Stretch activated channels are normally closed to ion flow, however when stretch occurs on the membrane, the channel opens and Ca^{2+} ions are allowed to enter the cell. Stretch inactivated channels function in the opposite way. The stretch inactivated channels are closed to Ca^{2+} ion flow when the membrane is stretched but they are open to Ca^{2+} flow when there is no stretch applied to the membrane. It has also been suggested that cytoskeletal changes due to muscular dystrophies may also alter



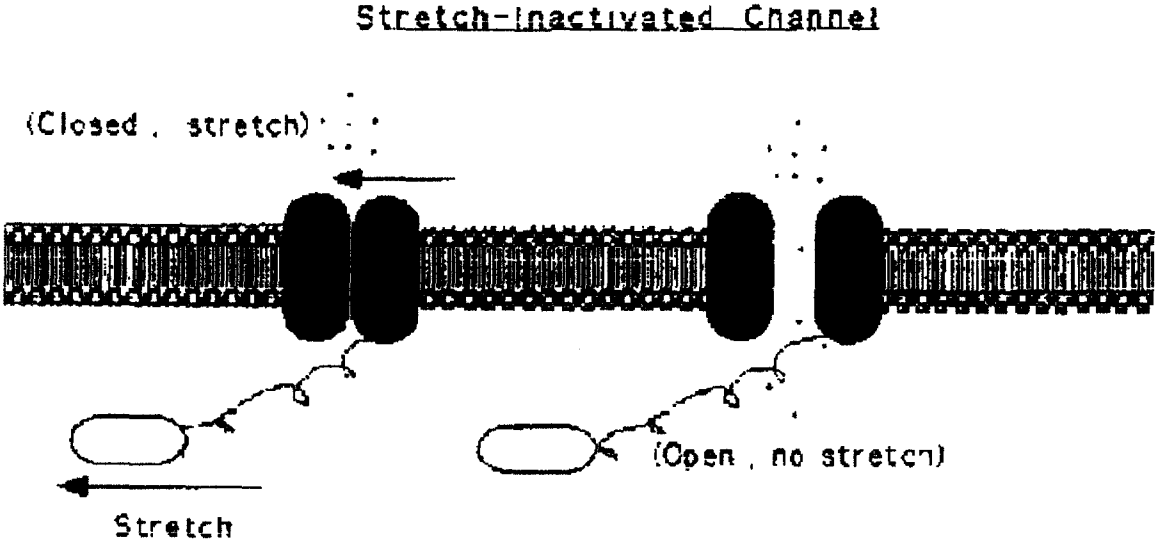


Figure courtesy of Dr. Gary Walker

mechanosensitive ion channel function, contributing to pathogenesis in muscular dystrophy and perhaps other neuromuscular disorders (Franco et al., 1990; Franco-Obregon et al., 1994).

There is a strong body of evidence presented in these pages to suggest that there are an abundance of anti-muscle antibodies in patients with myasthenia gravis and a thymoma. Several other authors have attempted to attribute muscle dysfunction to many of these muscle proteins. This study attempts to identify the specific muscle cell proteins that are being targeted in the autoimmune response of myasthenia gravis patients with a thymoma that may be responsible for rippling muscles. Identification of these proteins will lead to a better understanding of their role in normal muscle tissue function.

III. Materials and Methods

A. Experimental Design:

The experiments performed were designed to determine the differences in immunoreactivity between the sera obtained from several donor patients. Experimental patient donors include one who has been clinically diagnosed with rippling muscles and a thymoma and two other patients with rippling muscles and myasthenia gravis without a thymoma. Control patients who have been diagnosed with myasthenia gravis and a thymoma but are negative for rippling muscles. I have also used sera obtained from patients with myasthenia gravis but without a thymoma to serve as positive controls for myasthenia gravis and negative controls for thymoma and rippling muscles. Sera from healthy control patients to served as a negative control.

Whole human skeletal muscle was used as the antigen source for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two dimensional electrophoresis, western blotting and immunocytochemistry. Rat skeletal muscle was also examined as a source of antigens to eliminate both the hazards and difficulty in obtaining quantities of human tissue for study.

B. Preparation of SDS-PAGE samples:

SDS-PAGE samples of whole skeletal muscle were prepared by homogenizing approximately 0.5 g skeletal muscle tissue in a tissue

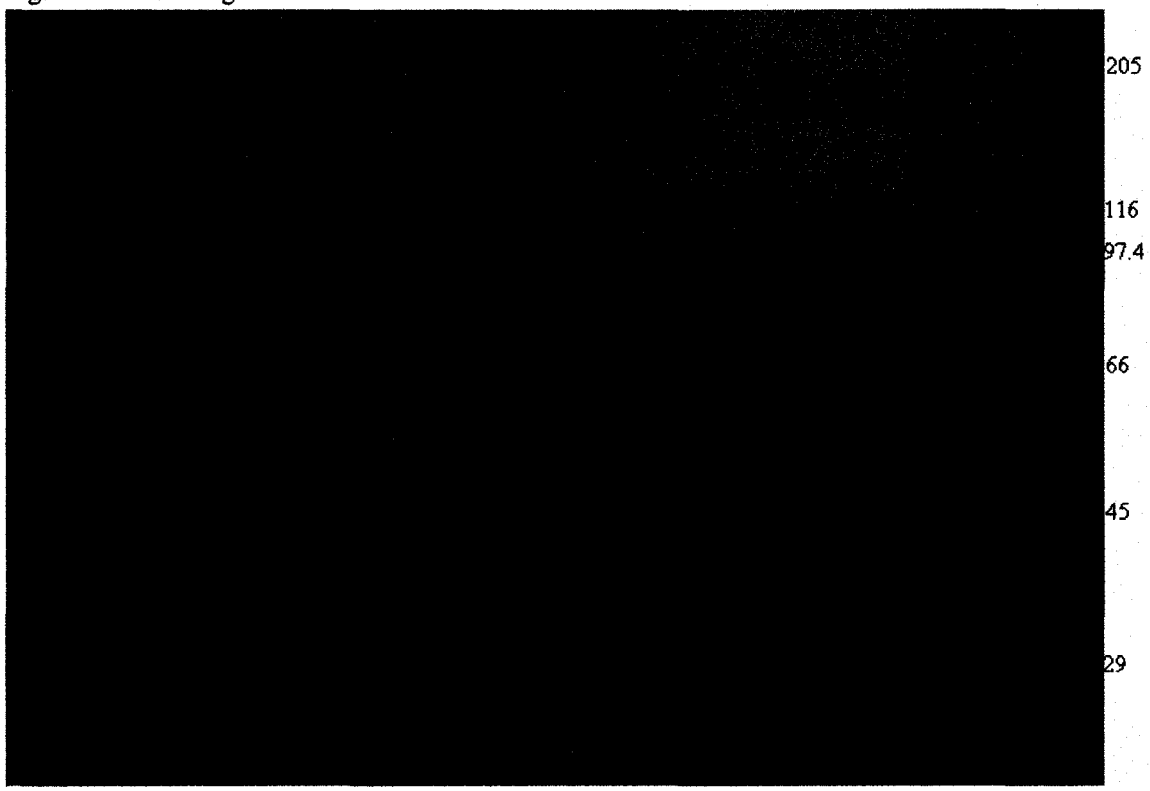
homogenizer. This homogenized tissue was added to 1 ml of 1x SDS sample buffer containing 12.5% glycerol, 50 mM TRIS (pH 6.8), 5% 2-mercaptoethanol and 2.3% SDS. The SDS in the sample buffer gently denatures proteins and gives them a large amount of negative charge. This results in all proteins having roughly the same charge to mass ratio, leaving mass as the only factor that determines their rate of mobility through the acrylamide gel. The samples were then heated to 100°C for two minutes to denature the proteins and allow SDS to bind to the protein molecules. Muscle samples were centrifuged in a microcentrifuge at 10,000 rpm for 10 min. to sediment myofibrils and other insoluble materials. The pellet was discarded and the supernatant was stored at -20°C (figure 4 shows a 7.5% acrylamide gel mini-slab gel that was performed to determine the optimum load size for a human skeletal muscle sample).

Tissue from rat and human skeletal muscle were prepared to create a sample rich in sarcoplasmic reticulum proteins. A rat was sacrificed and 25 g of skeletal muscle from the hindquarters was removed from the rat. Human skeletal muscle was obtained courtesy of Dr. Carl Ansevin. The muscle tissue was homogenized in a blender for 2 min in a solution containing 100 mM KCl, 2mM EDTA, 2.5 mM KH_2PO_4 and 2.5 mM K_2HPO_4. Next the myofibrils were sedimented by centrifuging the sample at 3300X g for 30

Lane #

1 2 3 4 5 6 7 8 9 10 11 12 13 14

High Molecular Weight



Low Molecular Weight

min. The supernatant of this solution was then centrifuged at 3500X g for 45 min to remove the mitochondria. The resulting supernatant was then ultracentrifuged with a Beckman L7 ultracentrifuge at 44,000X g for 60 min. The pellet from this step was resuspended in 4.8 ml of a solution containing: 1M sucrose and 50 mM KCl. The pellet was dispersed with a tissue grinder and centrifuged at 4500 X g for 15 min. The supernatant was decanted and placed into 3.9 ml of a solution made with 15 ml ddH2O, 2M KCl and 0.24 ml of 0.1M ATP. This is then centrifuged at 80,000 X g for 90 min. The pellet from this step was resuspended and washed twice with 10 ml of 0.1 M KCl. The pellet was resuspended for each wash and centrifuged at 80,000 X g for 60 min. The pellet was finally resuspended in 1 ml of 0.1 M KCl solution and stored at -20°C

C. SDS-PAGE:

Muscle protein analysis was carried out by SDS-PAGE using minislab gels (Matsudiara et. al. 1978).

1. Preparation of minislab gels:

Minislab resolving gels were prepared in a gel pouring chamber at the following concentrations of acrylamide: 12%, 10% and 7.5%. These resolving gels were prepared in batches of 12. The 12% gels were made by the addition of 6.4 g glycerol, 24 ml resolving gel buffer (made from the

addition of 18.17 g of TRIS, 8.20 ml 3M HCl and 0.40 g SDS to 100 ml ddH_20), 38.4 ml acrylamide stock, 240 µl ammonium persulfate (APS) and 96 µl TEMED to 25.6 ml ddH2O. The 10% gels were made by the addition of 6.4 g glycerol, 24 ml resolving gel buffer, 32 ml acrylamide, 240 µl APS and 96 µl TEMED to 32 ml ddH2O. The 7.5% gels were made by the addition of 6.4 g glycerol, 24 ml resolving gel buffer, 24 ml acrylamide, 240 µl APS and 96 µl TEMED to 40 ml ddH2O.

The acrylamide solution was then poured between glass plates separated by 0.5 mm spacers. This solution was overlaid with resolving gel buffer that had been diluted 4:1 by ddH2O. These gels were allowed to polymerize overnight at room temperature. The gels were removed from the pouring chamber, the overlay solution was discarded and the gels were stored in an airtight container at 4°C for a period of up to two months.

Before use, the resolving gel was overlaid with stacking gel. This stacking gel was made by the addition of 1.1 ml stacking gel buffer (prepared from stock consisting of 6.05 ml TRIS, 0.4 g SDS, 29.1 ml 2M HCl, and ddH2O to 100 ml), 750 µl acrylamide, 30 µl APS and 11 µl TEMED. The stacking gel was quickly poured between the glass plates and a sample well comb was placed in between the glass plates to allow space for the samples (figure 5 illustrates the process of pouring of minislab gels).

Figure 1.

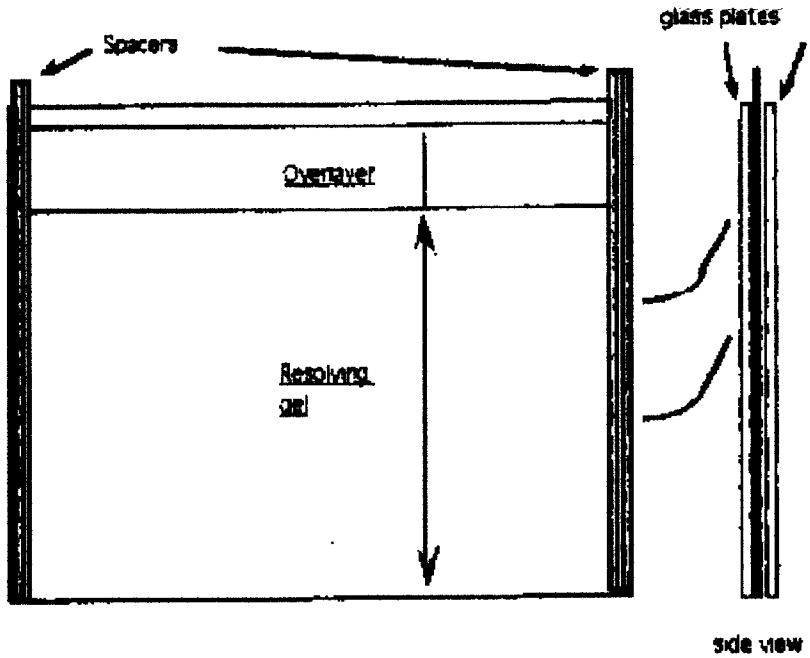


Figure 2.

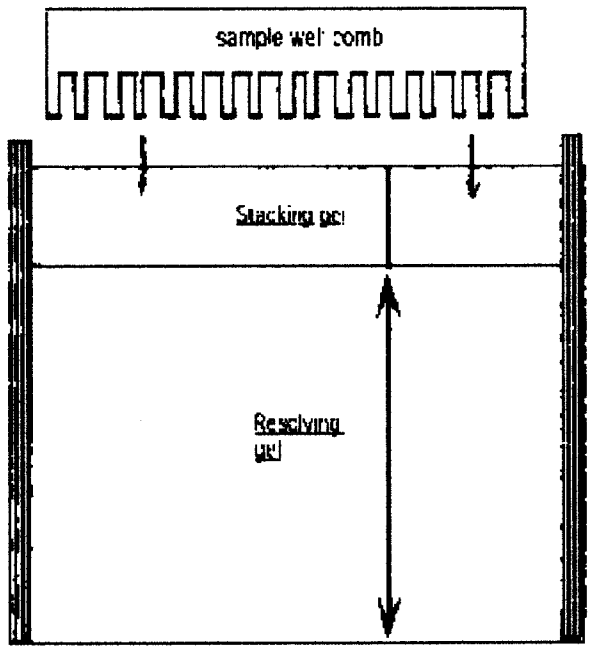


Figure courtesy of Dr. Gary Walker

Minislab gel sandwiches were placed in a gel chamber where they were loaded with sample (load size depending on the sample used). The upper and lower chambers were filled with SDS electrode buffer. This electrode buffer was made by the addition of 9.075 g TRIS, 43.26 g glycine and 3.00 g SDS to 3 liters of ddH2O. The electrode buffer was used at a pH of 8-8.5. The negative electrode was attached to the upper half of the chamber and the positive electrode was attached to the bottom half of the chamber so that the negatively charged proteins would run to the bottom of the gel in the positive chamber. Minislab gels were run at a constant current of 0.025 amps for a period of 1.5 hours. The glass plates were pried apart and the gels were removed for further analysis by either coomasee staining or western blotting.

2. Coomassie staining of minislab gels:

The minislab gels were placed in a solution of 0.2% Coomassie Brilliant Blue R-250, 45% methanol and 10% acetic acid. The gels were stained for a period of about 15 minutes. The gels were then destained in a solution of 45% methanol and 10% acetic acid for a period of one hour with frequent changes of the destaining solution. The stained gels were dried between two sheets of dialysis membrane and analyzed for total protein distribution and optimal load size for later samples.

D. Electrophoretic transfer of proteins:

This procedure was performed to transfer proteins that had previously been separated by SDS-PAGE, onto a support membrane that binds proteins for immunoblot analysis. SDS-PAGE minislab gels prepared as previously described were transferred onto sheets of PVDF membrane by the use of a semiwet blotting apparatus from Idea Scientific "Genie".

The tray was assembled from the bottom up. First the negative electrode was placed into the transfer chamber followed by a bubble screen. The next layer in the transfer chamber consists of two Scotch Brite pads. The chamber was then filled to the level of the Scotch Brite pads with transfer buffer. This transfer buffer consisted of 57.6 g glycine, 12.1 g TRIS, 800 ml methanol and 3200 ml ddH2O. A piece of filter paper cut to the size of the Scotch Brite pads was placed into the chamber and the bubbles were all squeezed out of the chamber to ensure a clean transfer of proteins. The minislab gel was gently placed on top of the filter paper to ensure that there were no bubbles between the layers. The PVDF membrane was then prepared for transfer by first wetting it in methanol, then by rinsing the sheet in distilled water. This sheet was then carefully placed on top of the gel. A second piece of filter paper was placed over the PVDF. Two more Scotch

Brite pads were placed into the chamber. A second bubble screen was also placed in the chamber, followed by the positive electrode. This chamber was then closed with a Plexiglas sheet and placed into the running chamber. The whole apparatus was tilted into running position. The positive and negative electrodes were connected to the power supply. Proteins were transferred at a constant 25 volts for 1 hour.

E. Western Blotting:

Western blotting is a process of using protein specific antibodies to selectively stain a membrane that contains the proteins of interest.

1. Preparation of Sera:

Experimental sera were obtained from a clinical setting. Upon diagnosis, blood was donated by the test and control subjects and immediately centrifuged for 10 min in a clinical centrifuge to separate the whole blood fraction from the serum fraction. The samples were immediately frozen at -80°C for storage. The sera was numbered to maintain patient anonymity (Table 1 describes patients by number and symptoms) .

2. Blocking of PVDF:

PVDF sheets were placed in a blocking solution in order to prevent non-specific binding of antibodies to the PVDF membrane. The sheets were

Patient #	Patient Symptoms
1	Rippling muscle/thymoma/MG (No rippling apparent)
1	Rippling muscle/thymoma/MG (Rippling symptoms has returned)
9	Rippling muscle/MG
13	Rippling muscle/MG
3	MG/thymoma
4	Healthy control
5	MG/ocular
6	MG/mild
7	MG/mild
8	Malignant hyperthermia
10	MG/post-thymoma
12	MG/mild

Table 1

placed in a solution containing 5% powdered non-fat milk and TBS-T TRIS buffered saline containing 0.5 M NaCl, 20 mM TRIS, 2% Tween 20 and adjusted to a pH of 7.3. The PVDF sheet was then left in the blocking solution for a period of one hour.

3. Primary Antibody Application:

The previously blocked PVDF sheets were placed in a 1% blocking solution made by diluting the 5% blocking solution four to one. The patient's sera was used as the primary antibody by adding it to the 1% blocking solution at a concentration of 500 to 1 and allowed to incubate for one hour. The primary antibody was removed from the solution by washing the PVDF membrane three times with TBS-T.

4. Application of Secondary Antibody:

The secondary antibodies we used were goat, anti-human antibodies obtained from SIGMA™. We used both Fc specific and Fab specific secondary antibodies that were labeled with the enzyme horseradish peroxidase. The secondary antibodies were applied to the PVDF blot by the same procedure as the primary antibody except at a 1:3000 concentration. The unbound secondary antibody was then rinsed three times with a solution of TBS to remove the tween detergent before the detection phase.

5. Chemiluminescent Detection:

The detection of bound antibodies was performed by the addition of a chemiluminescent substrate for the horseradish peroxidase labeled secondary antibody to react. For our studies, the substrate used was PIERCE Super Signal™ ULTRA. 2ml of substrate was applied to the membranes and allowed to react for a period of 2 minutes. The PVDF membrane was then placed in between two sheets of clear plastic. Care was taken to avoid the occurrence of bubbles between the membrane and the sheets. In the darkroom, the membrane was exposed to light sensitive paper by layering the paper over the plastic. The first exposure was for 30 seconds with subsequent exposures being either longer of shorter depending on the amount of chemiluminescent signal.

F. Two Dimensional Electrophoresis:

Two dimensional electrophoresis is a means of separating proteins on the basis of their isoelectric point (first dimension) and by protein mass (second dimension). This tool is important to us since many proteins have a similar mass, but no two have the same mass and isoelectric point. This in theory allows the separation of proteins that have only a single amino acid difference in their composition (O'Farrell et. al., 1975).

1. Preparation of samples for 2D Electrophoresis:

Samples of human skeletal muscle weighing approximately 0.5 g were frozen and lyophilized overnight to remove all tissue water. The freeze dried muscle was placed in 500 µl of lysis buffer made by the addition of: 5.70 g urea, 2.00 ml 10% NP-40, 2.00 ml 2 -mercaptoethanol, pH 6-8 ampholines, pH 3-10 ampholines, to 2.66 ml ddH_2O. Ampholines are molecules that have both a positive and negative charge, useful in setting up a charge gradient in the first dimension tubes. The samples are stored at
-80°C.

2. Preparation of First Dimension (IEF) Gels:

First dimension gels were poured in Fisher disposable micropipetes and referred to as "tube gels" or “tube worms”. These gels were prepared fifteen at a time in a conical centrifuge tube that had been inverted and had had the tip cut off. IEF gel is prepared by the addition of 5.50 g of urea, 1.33 ml acrylamide stock, 2.00 ml 10% NP-40, 0.40 ml pH 6-8 ampholines, 0.10 ml pH 3-10 ampholines, 20.0 µl 10% ammonium persulfate, 14.0 ul TEMED to 1.97 ml ddH2O. 4ml of the IEF gel was placed into the tube. This was then overlayered with ddH_2O to push the gel into the tubes. Enough ddH2O was used to push the gel up to the 200 µl mark in the tubes.

The first dimension tube gels were placed in a gel chamber where 20µl of sample was loaded into the tubes. Sample overlay solution made in 25 ml quantities (by the addition of 13.0 g urea, 0.5 ml pH 6-8 ampholines, 0.125 ml pH 3-10 ampholines, to 14.3 ml ddH2O) was placed over the sample in equal volumes. The gel chambers were next filled with anode (top) solution made by the addition of 1.69 ml of H3PO4 (85%) to 2500 ml ddH2O and cathode solution(bottom) made by the addition of 1.60 g NaOH to 2000 ml ddH2O. The samples were first pre-focused for 45 min (15 min. at 200 V, then 30 min. at 300 V) to allow the ampholines to establish the pH gradient in the tube gel. Samples were run for 4800 V/hr (300 V for 16 hours). Tube gels were carefully forced from the tubes and placed in 1ml 1x SDS sample buffer (made from the addition of 1.25 g glycerol, 0.76 g TRIS, 5.00 ml 2-mercaptoethanol, 2.30 g SDS and 0.5M HCl to 71.0 ml ddH2O) for 30 min. The tube gels were frozen and stored at -80°C.

3. Preparation of Second Dimension Gels:

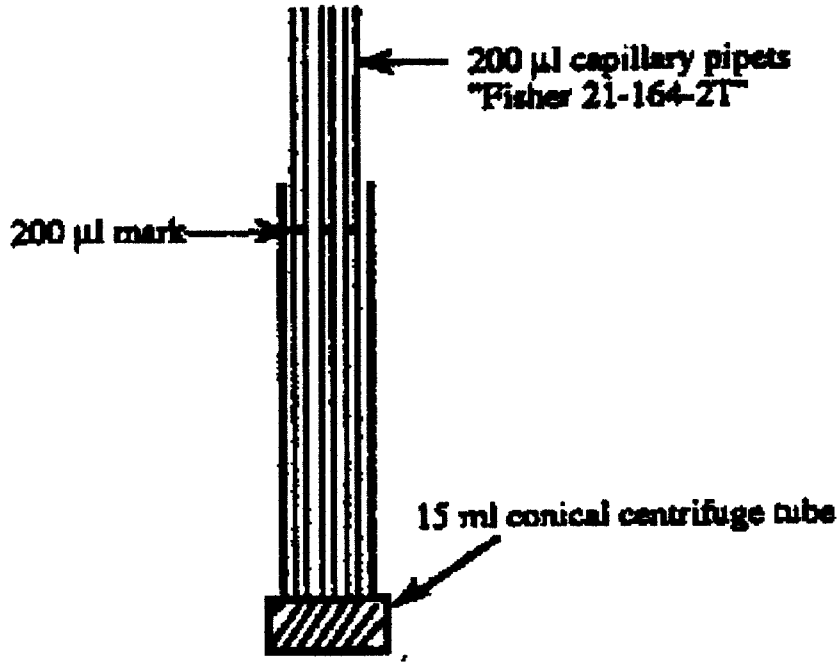
7.5% acrylamide gel solution was prepared as previously described and poured between two glass plates (one of the glass plates is two centimeters shorter than the other). The resolving gel was poured to a level 1.5 cm lower than the top of the shorter of the glass plates. Stacking gel prepared as previously described was poured over the resolving gel. A spacer was used

at the top of the stacking gel to preserve space for the tube gel. The tube gel was thawed and placed on a strip of parafilm for ease of manipulation. The excess fluid was removed from the tube gel and the tube gel was straightened and manipulated to the edge of the parafilm. The tube gel was imbedded in a solution of hot agarose (3%) at the top of the stacking gel. A glass probe was run along the edge of the tube gel worm to be sure that good contact between the worm and the stacking gel was achieved. The agarose was allowed to solidify before placing the minislab gel into the electrophoresis apparatus. The apparatus was filled with SDS electrode buffer (previously described), and 2 drops of tracking dye were added to the upper chamber of the apparatus to track the travel of the lightest peptides down the SDS-PAGE gel. The gel was electrophoresed at a constant current of 0.25 amps for 1-1.5 hours. The gel was then either stained with coomasee as described or it was transferred to PVDF for western blotting as described (figure 6 illustrates the pouring of 2D gels).

G. Immunocytochemistry:

1. Preparation of Tissue Sections:

The human skeletal muscle tissue sections that we used were obtained from Dr. Ansevin (courtesy of the histologist at St. Elizabeth's hospital). They were placed on glass slides and frozen immediately after sectioning and



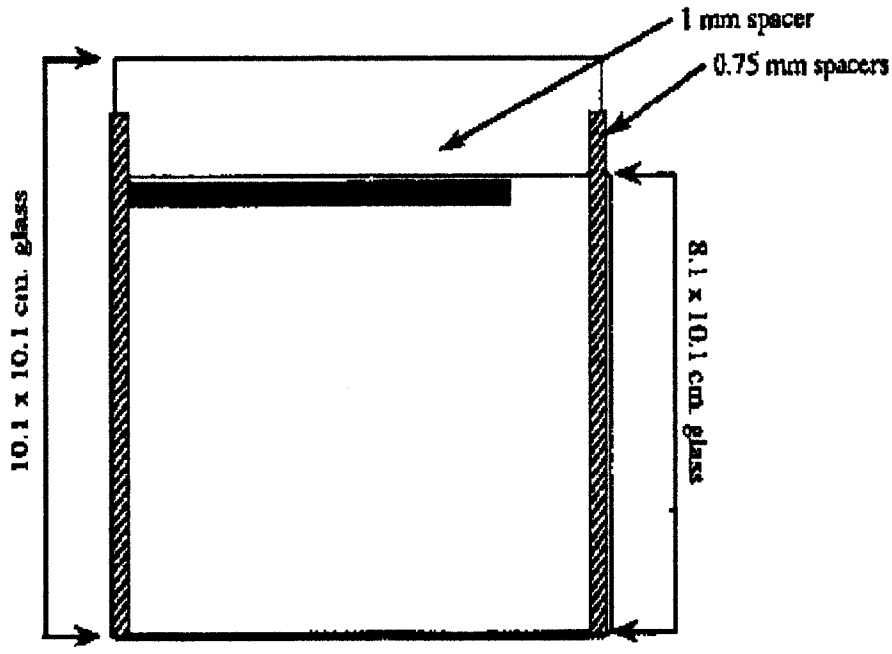


Figure courtesy of Dr. Gary Walker

stored at -80°C. These tissue sections were fixed in a 3.75% formaldehyde solution for 10 minutes prior to staining.

2. Cell Staining:

The muscle tissue sections were incubated with primary antibodies to identify cellular proteins that were bound by the patient's sera acting as the primary antibody. The secondary antibody used was a goat anti-human antibody specific for the Fab portion of human IgG, bound to a fluorochrome for detection of primary antibody in a fluorescent microscope. The sections were initially incubated for 1 hour in a blocking solution containing 5% powdered non fat milk and 0.05% Triton-X 100 detergent to slightly solubilize cell membranes in TBS (previously described). The primary antibody was then applied at a 1:500 concentration in a 1% blocking solution made by diluting the 5% solution 4:1 with TBS. After one hour the tissue sections were rinsed three times in TBS to wash away any unbound primary antibody. The secondary antibody was then applied at a 1:3000 concentration in a 1% blocking solution as described earlier. The tissue sections were again rinsed three times with TBS. The tissue sections were treated with anti-oxidant to act as a preservative, covered with a glass coverslips and sealed with nail polish. The slides were stored at 4°C, prepared slides were then photographed at full fluorescence with an Olympus

35mm camera (mod. # C-35AD-4) on an Olympus microscope equipped with a UV epi-fluorescence (mod. # BH-2).

IV. Results

Figure 7 shows a western blot autoradiograph that exhibits a large amount of non-specific antibody binding. This blot shows a large signal to noise ratio that obscures the immunoreactive bands. It is very difficult to obtain any useful data from an extreme example such as this. This problem was overcome after initial blotting experiments.

A western blot from the goat anti-human Fc specific secondary antibody is illustrated in figure 8. These blots were performed on a 7.5% acrylamide minislab gel, in which 5µl of sample was placed in each lane. Lane 1 shows a western blot using patient 1's sera (MG/thymoma/rippling muscle) as the primary antibody at a 1:500 concentration and Fc specific goat anti-human secondary antibody at a 1:3000 concentration. Immunoreactivity in lane 1 to relatively high molecular weight antigen at around 400 kDa is observed. Immunoreactivity is also exhibited to an intermediate and a relatively low molecular weight antigen at around 97 kDa and 77 kDa, respectively. Lane 2 shows a western blot autoradiograph that used goat anti-human secondary antibody at a 1:3000 concentration alone. Immunoreactivity on this blot is seen in the 77 kDa to 97 kDa range. Lane 3 shows a western blot autoradiograph that used only secondary antibody at a

1:500 concentration. Immunoreactivity is exhibited to antigen in the 77 kDa range indicating non-specific labeling

Figure 9 shows the results of a western blot analysis of sera from several patients. Human skeletal muscle prepared by the same protocol as the enriched rat skeletal muscle served as the sample in each lane. All primary antibody concentrations were 1:500 and secondary antibody in all cases was Fab specific goat anti-human at a concentration of 1:3000. Lane 1 shows Bio-Rad prestained molecular weight standards. Lanes 2-8 shows the results of western blot autoradiographs. Lane 2 reports the results with patient 9's sera as primary antibody (MG/rippling muscles). Immunoreactivity is observed in the 48 kDa range and the 77 kDa range. Lane 3 reports the results with patient 13's sera as primary antibody (MG/rippling muscles). Immunoreactivity is observed at the same range (48 kDa and 77kDa) as in lane 2. Lanes 3, 4 and 7 reports the results from patients with MG. Immunoreactivity in those samples is observed at around 48 kDa and around 77 kDa. Lane 5 shows the results using a thymoma patient's sera. Immunoreactivity in that sample is also observed at approximately 48 kDa and 77 kDa. Lane 6 shows the results using patient 10's sera (MG/thymoma). Immunoreactivity is again present at around 48 kDa and at 77 kDa. Lane 8 reports the results of patient 12's sera (mild MG).

Immunoreactivity is only present at around 77 kDa. A control lane using only secondary antibody is also performed alongside these autoradiographs (data not shown), no immunoreactivity was observed in the control.

Antisera from rippling muscle patients recognized unique muscle antigens (figure 10). Lane 1 shows Bio-Rad prestained molecular weight standards on a coomassee stained PVDF membrane. Lane 2 shows total human skeletal muscle protein distribution on a coomassee stained PVDF membrane. Lanes 3-7 reports the results of western blot autoradiographs using rippling muscle patient's sera as the primary antibody at a 1:500 concentration and Fab specific goat anti-human secondary antibody used at a 1:3000 concentration. Lane 3 reports the results of patient 1's sera (MG/thymoma/rippling muscle) collected at a period when rippling symptoms had disappeared but MG is severe. Trace immunoreactivity to a peptide with a molecular weight around 400 kDa and to a peptide with a weight around 97 kDa is also observed. Lane 4 reports the results from the same patient with primary antibody serum obtained at a time when the rippling muscle symptoms had returned. Immunoreactivity to a relatively high molecular weight (around 400 kDa peptide) is observed as well as immunoreactivity to intermediate (around 200 kDa) and low molecular weight peptides (around 97 kDa). Lane 5 reports the results of patient 9

(MG/rippling muscles). Immunoreactivity in this autoradiograph is observed to be very similar to lane 4, with bands at around 400 kDa, 200 kDa and 97 kDa. Lane 6 used a control patient's (MG/thymoma) sera as the primary antibody. In this lane immunoreactivity is only observed at around 97 kDa. Lane 7 reports the results of a control patient with MG and a thymoma. Immunoreactivity is observed to a high (>400 kDa peptide) and a low (approximately 97 kDa) peptide.

Western blot analysis identified several possible high molecular weight antigens present in the stacking gel (figure 11). Lane 1 reports the results of patient 1's sera (MG/thymoma/rippling muscles) at a period when rippling symptoms were not present. The autoradiograph shows that there is immunoreactivity present to high molecular weight antigen (>400 kDa) in the stacking gel and immunoreactivity to high molecular weight antigens in the resolving gel. Lane 2 shows the results of patient 1's sera (with rippling symptoms). Immunoreactivity to high molecular weight antigen is again observed in the stacking gel and to high molecular weight antigen in the resolving gel. Immunoreactivity in the second sample (with rippling symptoms) appeared to be more intense at the same exposure level. Lane 3 reports the results of patient 9's sera (MG/thymoma). Immunoreactivity is again present to high molecular weight antigen in the stacking gel and to a

high molecular weight antigen in the resolving gel. Lane 4 reports the results of patient 13's sera. Only trace immunoreactivity to the high molecular weight antigen in the resolving gel is observed. Lane 5 reports the results of patient 7's sera (MG mild). Only trace immunoreactivity to an intermediate weight antigen is observed. Lane 6 shows that there is no immunoreactivity in patient 5's (MG mild) sera. Lane 7 reports the results of patient 3's (MG/thymoma) sera. Immunoreactivity is observed in the stacking gel and to a high intermediate and low molecular weight antigens in the resolving gel. Lane 8 shows the results of patient 10's sera (MG/thymoma). Trace immunoreactivity was observed in the stacking gel and to high molecular weight antigen in the resolving gel. Lane 9 reports the results of patient 12 (MG mild) sera. No immunoreactivity is observed. Lane 10 is a control (secondary antibody only) lane. No immunoreactivity is observed using only secondary antibody.

Table 2 summarizes the results obtained by western blotting in figure 9 as well as western blotting on control patients (data not shown). There is a high incidence of immunoreactivity in the stacking gel and at a high molecular weight antigen in patients with rippling muscles when compared to patients with MG alone. Thymoma patients did show some immunoreactivity to antigen in the stacking gel and to a high molecular

weight resolving gel antigen. The thymoma patients did not display as much immunoreactivity intensity as the rippling muscle patients at these molecular weights.

Two dimensional gel electrophoresis of human skeletal muscle increases the resolution of individual peptides (figure 12). Separation of proteins on the basis of both iso-electric pH and molecular weight indicates the complexity of proteins present in muscle.

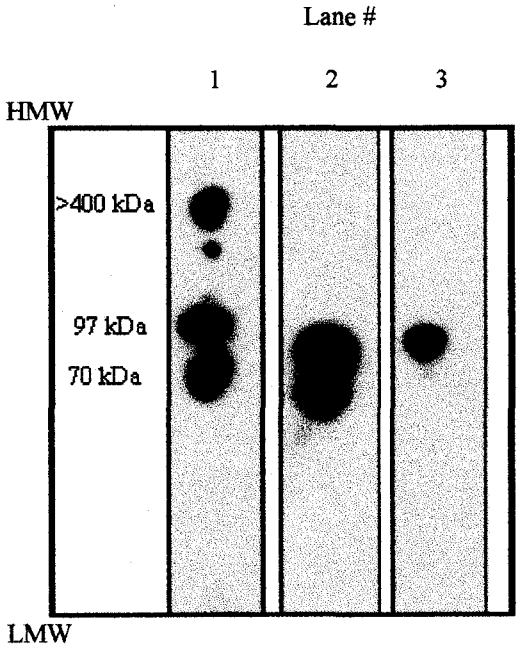
Figure 13 shows the results of a western blot analysis of two dimensional IEF gels revealing a complicated pattern of antigen recognized by antibody. Patient 1's serum was used as the primary antibody probe in this blot of a 2D gel. Immunoreactivity is observed at an intermediate molecular weight toward the basic region of the peptide constellation. This figure also includes a coomassee stained PVDF membrane showing the distribution of proteins in this sample.

There is considerable variation in 2D labeling patterns between patient's sera and individual blots (figure 14). Patient sera numbers: 1, 13 and 6 were compared. Image 1 reports the results of patient 1 sera (MG/thymoma/rippling muscles). The image shows that there is immunoreactivity to relatively high molecular weight peptides that are toward the basic end of the constellation. The autoradiograph performed with

patient 13 (MG/rippling muscles) sera shows that there is immunoreactivity to intermediate molecular weight peptides. Image 3 shows the results obtained with patient 6 (MG). Immunoreactivity is observed to intermediate molecular weight peptides.

Immunocytochemistry performed on human skeletal muscle tissue indicates cell surface localization of antigen (figure 15) The images shows a tissue section stained by patient 11's sera at a 1:500 concentration as the primary antibody. A fluorescent Fab specific goat anti-human secondary antibody was used at a 1:3000 concentration. Patient 1's sera shows labeling of antigen along the cell margin (figure 15; A&B). In many areas the labeling shows striational banding (arrows). Secondary antibody shows low level diffuse labeling uniformly over the cells (figure 15; C&D). Sera from patient 9 shows antibody binding along the cell margin and some antibody possibly binding to extracellular matrix proteins (data not shown).

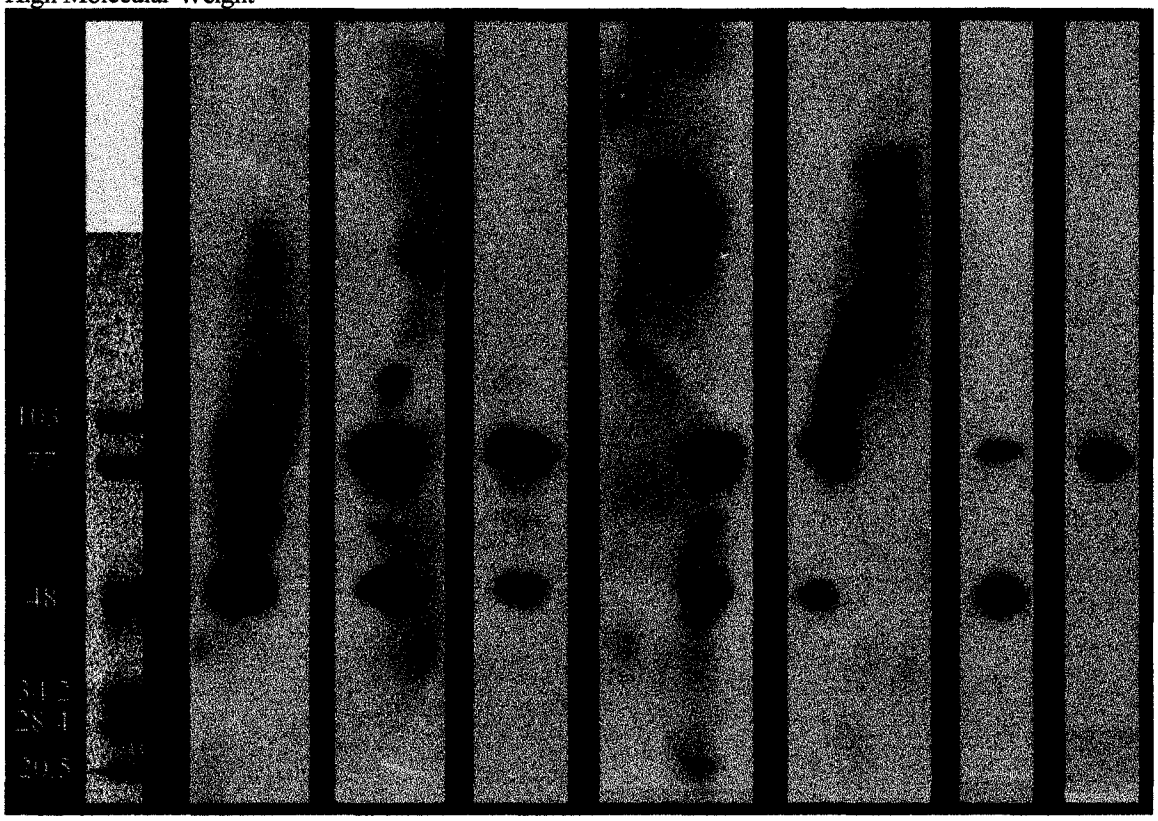
Western Blot Autoradiograph


Lane #
1
2
3
HMW
>400 kDa
97 kDa
70 kDa
LMW

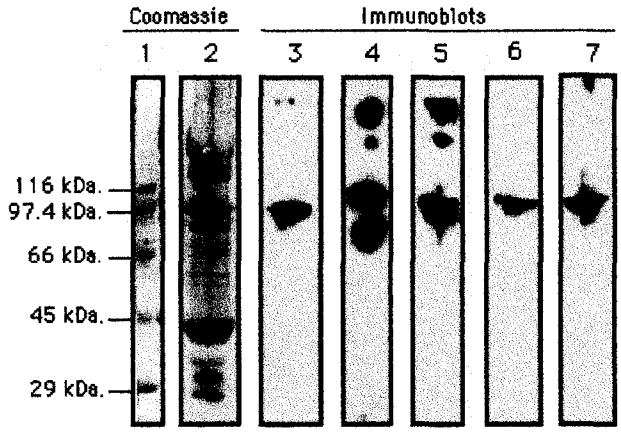
Lane #

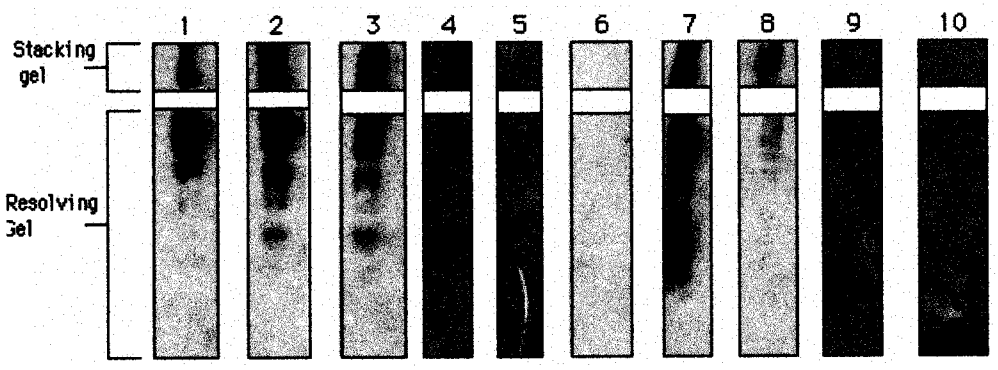
1 2 3 4 5 6 7 8

High Molecular Weight



Low molecular Weight

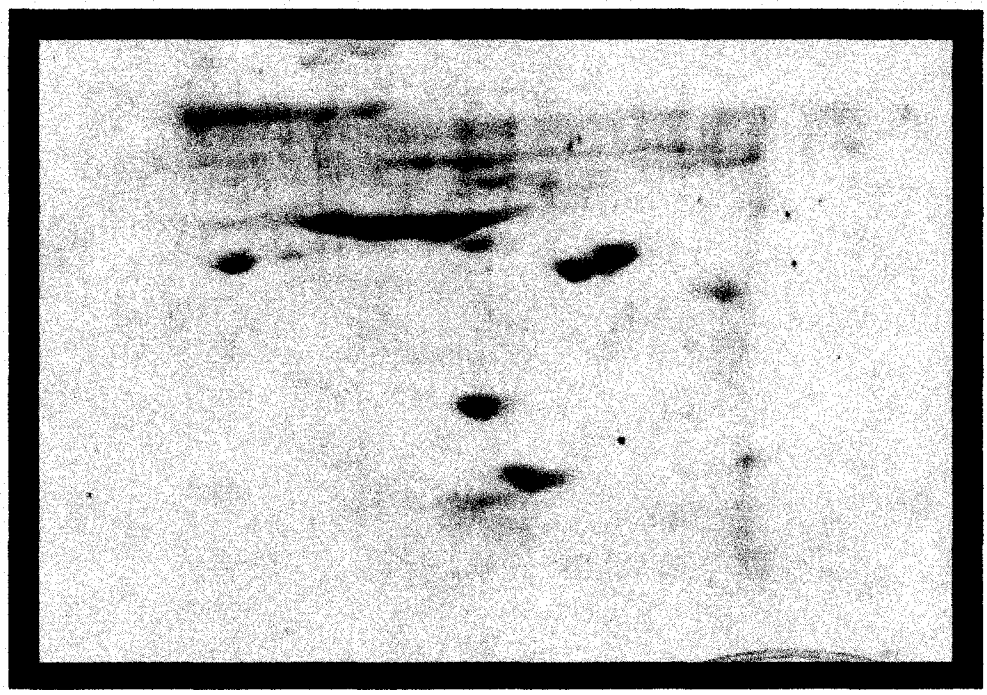

Coomassie
Immunoblots
1
2
3
4
5
6
7
116 kDa.
97.4 kDa.
66 kDa.
45 kDa.
29 kDa.


1
2
3
4
5
6
7
8
9
10
Stacking gel
Resolving Gel

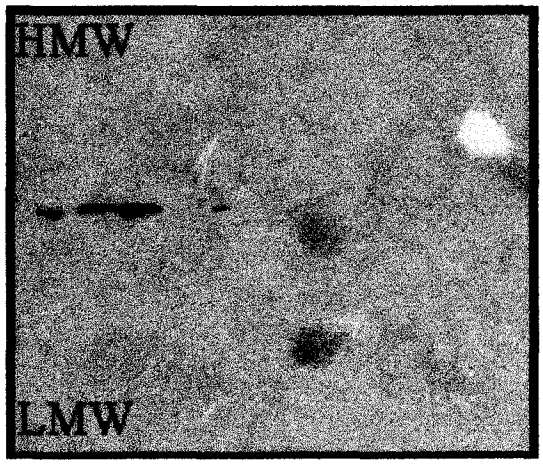
Summary of Auto-antibody Immunoblot Patterns

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

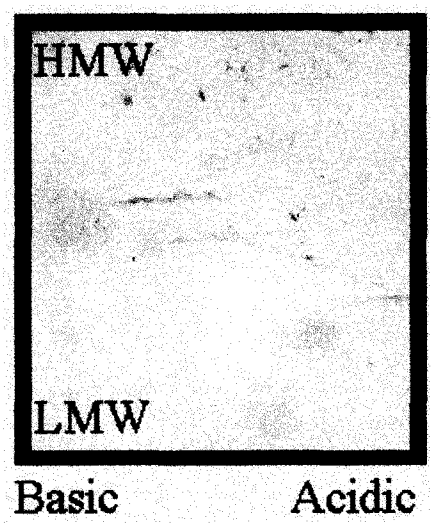
Coomassee stained gel



Western Blot Autoradiograph
Patient # 1 Sera



Coomassee Stained PVDF Blot



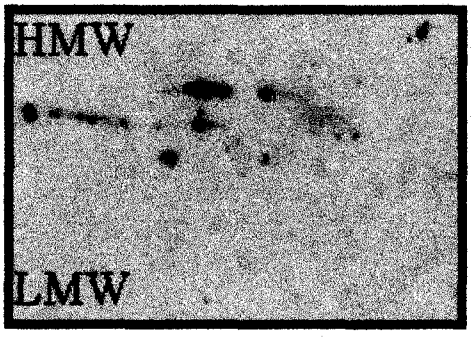
A

Western Blot Autoradiograph
Using Patient # 1 sera

Basic Acidic

B

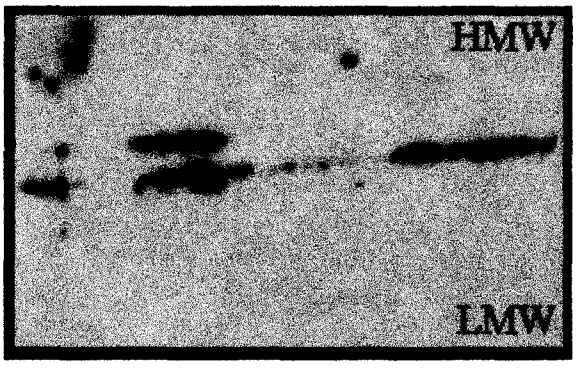
Western Blot Autoradiograph
Patient # 13 sera



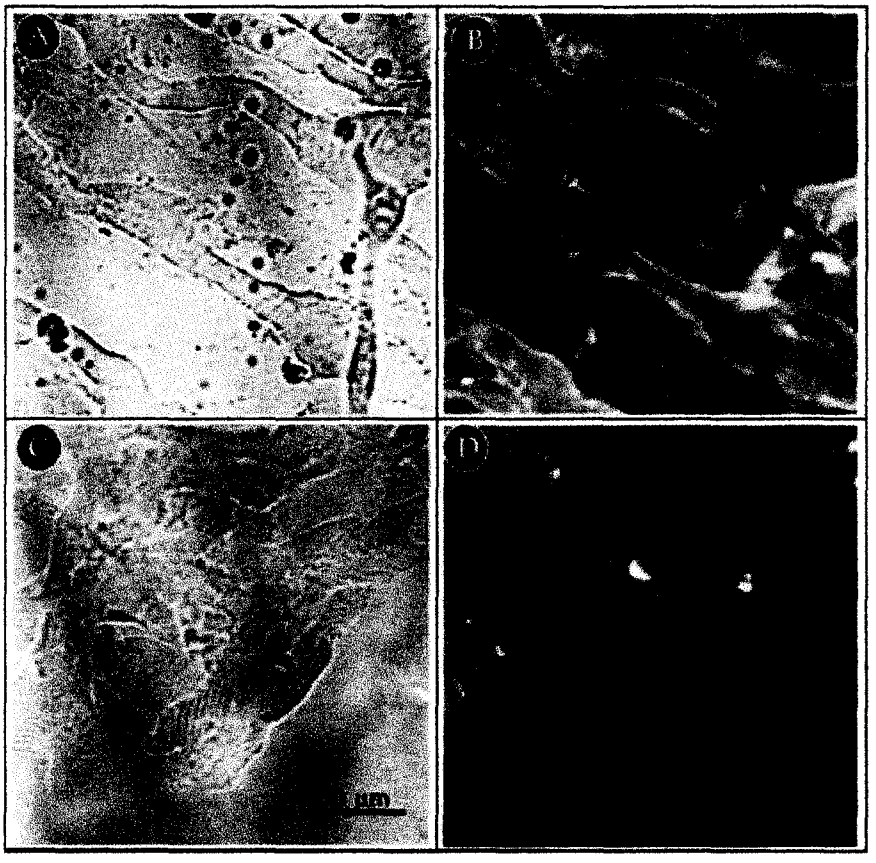
Basic Acidic

C

Western Blot Autoradiograph
Patient # 6 sera



Basic Acidic


A
B
C
D

V. Discussion

Rippling muscles associated with myasthenia gravis may be due to an autoimmune reaction caused by abnormal cell growth in the thymus (Ansevin et. al., 1996) It has been the goal of these studies to characterize the autoantibodies that may be causing the rippling muscle symptoms and to identify possible antigens in skeletal muscle responsible for rippling muscles.

Western blot data with patient sera (Figures: 8 and 9) show that there are high molecular weight peptides recognized in patients with rippling muscles that are not found in patients with myasthenia gravis alone. Figure 8 compared the differences in patient 1's sera at two different times. The first (lane 3) was when the rippling muscle symptoms were not apparent but MG was severe, the second (lane 4) was when the rippling muscles were severe but the MG was under control. There appears to be a difference in immunoreactivity between the samples. The western blot performed on the sera from a non-rippling time only had trace immunoreactivity to a high molecular weight antigen (around 400 kDa). The sera sample taken at a period of severe rippling muscles shows much more immunoreactivity to a high molecular weight antigen (around 400 kDa) as well as immunoreactivity to an intermediate molecular weight antigen (around 200 kDa). Both sera also show immunoreactivity to a relatively low molecular weight antigen

(around 77 kDa). This suggests that there is a component of the disease that could be manifested by the autoantibodies to the high or the intermediate molecular weight antigen. This is further confirmed in figure 8, lane 5 patient 9 (MG/rippling muscles). This patient also showed immunoreactivity to the high, intermediate and low molecular weight antigen at very similar molecular weight ranges. A third patient with rippling muscles showed little immunoreactivity on either blot (figures 8 &9). This may be due to the fact that the serum was obtained from Germany (unfrozen, via postal service), which may have caused degradation of the sample. The immunoreactivity displayed on the blots with these two sera show that there are bands at the high, intermediate and low molecular weights. The immunoreactivity at the high molecular weight suggests that this may play a direct or indirect role in the rippling symptoms, since it is present at only trace levels in patient 1 at a period of non-rippling and it is very prominent in the sera of the patient at a period of severe rippling, as well as in another rippling muscle patient (patient 9). In the control patients with myasthenia gravis alone, immunoreactivity in a weight range around 40 kDa-70 kDa, would be expected since acetylcholine receptor subunits all fall within this range. Immunoreactivity at the molecular weight of intact acetylcholine receptors, around 290 kDa, would also be expected (Changeux et. al. 1984). It is highly

unlikely that this protein would remain intact in SDS-PAGE. Little or no immunoreactivity was seen in MG control patients at any molecular weight. This may be due to two factors. One factor may be a loss of the antigen-antibody binding site with the denaturation (with subsequent loss of tertiary structure) of the proteins in the sample in preparation for SDS-PAGE. Another factor may be a low level of acetylcholine receptor in the skeletal muscle samples. Since acetylcholine receptors are concentrated only at the neuromuscular junction, there may be too little AchR in the sample to be detected with this technique.

The immunoreactivity at the high molecular weight in patients with rippling muscles may be ryanodine receptor, a Ca^{2+} release channel protein (Coronado et. al. 1994). The ryanodine receptor has a molecular weight of about 350 kDa and is thus within the molecular weight range of the high molecular weight bands recognized by antibodies from rippling muscle patients. It is unlikely that the ryanodine receptor is the causative agent in rippling muscles because it is found in the sarcoplasmic reticulum, not the sarcolemma. Therefore antibodies would have to cross the plasma membrane to cause an effect on the ryanodine receptor. It is much more plausible that the autoantibodies to ryanodine receptor are an effect of damage to the

muscle cell membrane and exposure of the ryanodine receptor to the immune system leading to autoimmunity.

A recent study by Marx et al., 1998 reported that there is an association between ryanodine receptor in the sarcoplasmic reticulum and dihydropyridine receptor (DHPR) in the T tubule membrane. It is believed that the surface membrane depolarization is sensed by the DHPR, which then triggers a the release of calcium from the SR through a mechanical link to the RyR. In light of this report, there is a possibility that the mechanical link between DHPR and RyR could be effected by an antibody. This disturbance could make the muscle cells more sensitive to mechanical activities like stretch and percussion, leading to the rippling symptoms.

The shared immunoreactivity patterns between rippling muscle patients may also suggest the involvement of autoantibodies to stretch-activated (SAC's) or stretch-inactivated channels (SIC's) (Ansevin et. al 1996; Gucharay et. al. 1984). The molecular weight of these is not known at this time, however the symptoms of rippling muscle are suggestive of this type of calcium channel in the skeletal muscle. Autoantibodies to these mechanosensitive channels may be responsible for rippling muscles by binding to the channel proteins, producing a conformational change in the proteins and causing the channels to leak Ca^{2+} into the cell.

percentage acrylamide gels to gain greater resolution of the high molecular weight antigen.

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HM HEALTH SERVICES
ST. ELIZABETH HEALTH CENTER

March 27, 1998

Carl F. Ansevin, M.D.
7417 South Avenue Extension
Boardman, Ohio 44512

RE: *IRB Approval # 98-008*

Dear Dr. Ansevin:

The following project was reviewed at the Institutional Review Board meeting held on March 25, 1998.

Title: Characterization of Auto-Antibodies Responsible for Rippling Muscles in Patients With Autoimmune Rippling Muscles and Myasthenia Gravis

Principle Investigator: Carl F. Ansevin, M.D.

The IRB has approved the above protocol and the informed consent document (with minor changes as specified below) for one year, expiring on March 25, 1999. This approval is contingent upon your making satisfactory arrangements with the Director of Patient Accounting, HM Health Services, according to HMHS regulations per the attached March 9, 1998 memo from Shelley Vazmina. Please contact Keith Alltop, Director of Patient Accounting, HMHS concerning appropriate billing procedures before you begin this clinical investigation. Also, please make arrangements with Pharmacy Services for appropriate handling of investigational drugs in your study, if applicable.

The IRB recommended the following changes to the informed consent document, versions b, b-1, b-2, b-3, b-4, and b-5. Please delete the word "extension" located after Dr. Watanakunakorn's phone number, the phrase should read ".....I may contact Dr. C. Watanakunakorn in the Office of Medical Research at St. Elizabeth's Health Center at (330) 746-7211."

This IRB is in compliance with the regulations of the FDA as described in 21 CFR parts 50 and 56. According to federal guidelines, all human research projects are approved for one year. If the project lasts for more than one year, an annual progress report must be submitted to the IRB with a request for reapproval.

If you have any questions, please do not hesitate to contact me by paging me through SEHC hospital operator.

Sincerely,

C. Watanakunakorn

Chatrchai Watanakunakorn, M.D., Chairperson
Institutional Review Board

CW/tf
attachment

Youngstown State University / One University Plaza / Youngstown, Ohio 44555-0001

MEMORANDUM

DATE: April 6, 1998

TO: Gary R. Walker, Biological Sciences

FROM: Eric Lewandowski, YSU Human Subjects Committee

RE: Protocol # 27-98

This is to confirm the Committee action taken at its meeting on Friday, April 3, 1998 with respect to the above protocol:

(1) Based on a preliminary review of the protocol materials which you provided, and our earlier telephone conversations regarding this project, I concur that the research under Human Subjects consideration is exempt from full Committee review under U.S. DHHS Category 4 exemption;

(2) the YSU Human Subjects Committee recognizes that the primary research conducted by Dr. Ansevin, as principal investigator, represent procedures, methods and activities independent of YSU Human Subjects jurisdiction, and, consequently, the approval of the YSU Committee only extends to those activities conducted by you, on-site, as described in your protocol;

(3) should the St. Elizabeth Institutional Review Board provide independent approval of all or a portion of your research, the YSU Human Subjects Committee will recognize the primacy of the St. Elizabeth IRB in approving those aspects of your research that are conducted under their jurisdiction.

ECL/

cc: Distribution

Youngstown State University / One University Plaza / Youngstown, Ohio 44555-0001

April 15, 1998

Dr. Gary Walker
Department of Biological Sciences
UNIVERSITY

RE: Human Subjects Research Protocol #27-98

Dear Dr. Walker:

The Human Subject Committee has reviewed the modifications you submitted for your protocol, HSRC #27-98 "Characterization of Auto-Antibodies Responsible for Rippling Muscles in Patients with Autoimmune Rippling Muscles and Myasthenia Gravis," and determined that your protocol now fully meets YSU Human Subjects Research Guidelines. Therefore, I am pleased to inform you that your project has been approved.

Any changes in your research activity should be promptly reported to the Human Subjects Research Committee and may not be initiated without HSRC approval.

Unanticipated problems involving risks to subjects should be promptly reported to the Human Subjects Research Committee.

We wish you well in your study.

Sincerely,

Eric Lewandowski
Administrative Co-chair
Human Subjects Research Committee

c: Dr. Paul Peterson, Chair
Department of Biological Sciences
Human Subjects Research Committee

Celebrating YSU 30 Years as a State University