The Immunofluorescent Localization Of Antigens Associated With Autoimmnune Rippling Muscles

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

SCHOOL OF GRADUATE STUDIES

YOUNGSTOWN STATE UNIVERSITY

AUGUST, 2002

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ABSTRACT

Rippling muscle disease was first characterized by Torbergsen et al. in 1975. The rippling phenomena were described as stretch and percussion activated wave like contractions, that roll through the large skeletal muscles of the body. These muscle "ripples" appeared to be electrically silent (showing no motor unit action potentials). This disorder has been shown to be autosomal dominant and heterogenetic (Ricker et al., 1989). In 1996 Dr. Carl Ansevin described a patient who exhibited rippling phenomena without any family history of neuromuscular disorder. The patient's rippling muscles were associated with the onset of myasthenia gravis and both disorders were effectively treated with immunosuppressive therapies. This led to the assertion that one form of rippling muscles is related to an autoimmune response to stretch activated Ca^{2+} channels (Ansevin et al., 1996). Our lab has shown that there are autoantibodies to high and very high molecular weight proteins in skeletal muscle, this immunoreactivity is not shown in patients with myasthenia gravis alone. Our lab has also demonstrated that rippling muscle patients also have autoantibodies to native proteins of very high molecular weight by immunoprecipitation from skeletal muscle (Walker et al., 1999). This project describes the cellular localization of rippling muscle autoantigen. Immunocytochemistry with cultured skeletal muscle myocytes show autoantibody reactivity with components of the T-tubular region of skeletal muscle.

Acknowledgements

I would like to take this opportunity to express my gratitude to my thesis advisor, Dr. Gary Walker. He showed me the patients of a saint, guidance and knowledge. He always gave me time to figure things out on my own and if I could not, he was there to lend a helping hand. I am truly blessed to have an advisor with the zest of life and enthusiasm for science to inspire my journey. We learn something from everyone who touches our lives. I have learned so much from Dr. Walker, not only about lab work and science but, life in general. Thank you very much to my committee members, Dr. Paul Peterson and Dr. David Asch, for your time, dedication and support on my project. Thank you to Dr. Carl Ansevin for providing me with tissue samples and clinical insight. Thank you to Keytam Awad for her time listening to my complaints about life and for her help with my project. Thank you to Tom Watkins for showing me around the lab and explaining things to me and also for being my beck and call computer consultant as well as your encouragement and support.

Thank you to the Biology department for providing me with the necessary materials to help me with this program. A special thank you to Pat, for listening to me and supplying me with materials. Anything I ever needed was always there for me (even if Pat had to find it for me).

I would like to thank all of my friends for tolerating my school schedule and their support as well as their encouragement.

I would like to thank everyone at Sharon Regional Health System for his or her endless switches to accommodate my school schedule. I would like to express my appreciation for the excellent advice from Dr. Cohen. Most of all, I would like to express my gratitude for the inspiration and support from the following people; Jonelle Bellin, Al Huntley, Harshad Desai, Angela Orras, Mary Moss, Stephanie Giscombe, Beth Pontius and Mary Ellen Marovich-Hematology Supervisor.

Since you become the person your parents raised, I would like to thank my parents for guiding my path in life and their encouragement as well as their love and support. Thank you to all of my grandparents for their wisdom, encouragement and support.

I am sincerely grateful for all the people in my life who have shaped the person I am today.

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INTRODUCTION

There are three different types of muscle tissue found in the body. The muscle tissue is distinguished by certain features as follows; the location, striations arranged in an alternating pattern of dense dark regions with less dense lighter regions, non-striated or the lack of striations, voluntary or conscious control for contraction and relaxation, involuntary or unconscious control for contraction and relaxation. The first type of muscle tissue is smooth muscle, located in the walls of hollow internal structures. Smooth muscle is characterized as non-striated and involuntary. The next type of muscle tissue is cardiac muscle. Cardiac muscle is characterized as striated and involuntary. The last type of muscle tissue is skeletal muscle. Skeletal muscle is usually attached to bone. The function of skeletal muscle is for movement of the skeleton. Skeletal muscle is characterized as striated and voluntary. There are three basic functions for any type of muscle. Muscle functions are movement, stabilization and heat production to maintain normal body temperature. Muscle tissue is excitable, responding to electrical signals also known as action potentials. Muscle tissue can contract and relax in response to one or more action potentials. Extensibility is another characteristic of muscle tissue that allows the one muscle out of the opposing pair to extend while the other one is contracted and shorter in length. The elasticity of muscle tissue allows it to return to the original conformation after contraction or relaxation (Tortora et al., 1993).

Muscle microanatomy

Ultra structure of muscle shows a highly ordered structure that specializes in contraction (Tortora et al., 1993). One muscle fiber consists of thousands of long, cylindrical cells. The sarcoplasm has light and dark bands or cross-striations. The darker regions are called anisotropic or A-bands and the light bands are called isotropic or I bands. Contractile elements in the skeletal muscle are named myofibrils. Myofibrils consist of three different myofilaments referred to as thick, thin and elastic filaments. A sarcomere is a specific arrangement of myofilaments in the myofibril. Z discs or lines are plate shaped areas containing dense material to separate each sarcomere. A-bands in the sarcomere stretch the entire length of the thick filament. I-bands, are less dense than A-bands, therefore they are lighter. I-bands in the sarcomere only contain thin filaments. Z discs are located in the middle of the I-bands. H zones are located in the middle of A-bands. H zones only contain thick filaments and are separated by the M line. Elastic filaments consist of a protein called titin or connectin. Titin is used to stabilize the thick filaments to the Z discs.

Myosin is a contractile protein. The tail of the myosin points toward the M line in the middle of the sarcomere. Thick filaments contain the myosin head or cross bridges. The thin filaments contain actin. Actin is also a contractile protein. Actin functions as a myosin binding site. Each actin molecule on the thin filament contains a myosin binding site to adhere to the myosin head of the thick filament.

Thin filaments contain troponin and tropomyosin which are regulatory proteins. The function of tropomyosin is to shield the myosin binding sites on the actin molecule in order to relax the muscle fiber. The muscle is stimulated to contract by the release of free calcium, from the calcium release channels in the sarcoplasmic reticulum, the calcium couples to the troponin on the thin filament. The calcium troponin complex alter the conformation of tropomyosin. This action uncovers the myosin binding site on the actin molecules. Actin, from the thin filaments, binds to the myosin head on the thick filaments allowing the thick filament to slide over the thin filament by the stimulation of the ATPase cycle resulting in the decrease in sarcomere length. After the contraction is complete the membrane potential returns to a resting potential which triggers the calcium release channels in the sarcoplasmic reticulum to close. The free calcium is pumped from the sarcoplasm to the sarcoplasmic reticulum by active transport from an ATP powered calcium pump. Next, the troponin-tropomyosin complex returns to normal and conceals the myosin binding sites on the actin, resulting in a relaxed muscle.

Regulation of muscle contraction

An elaborate cascade of events must occur in order for a skeletal muscle fiber to contract. The first event is a nerve impulse received at the axon terminal end of the motor neuron. The nerve impulse stimulates the release of acetylcholine into the synaptic cleft. Acetylcholine complexes to

the acetylcholine receptors on the motor end plate of the muscle sarcolemma. The coupling of acetylcholine to the acetylcholine receptor causes a conformational alteration, permitting free sodium ions in the sarcomere. This creates an action potential across the sarcolemma surface and into the middle of the sarcomere by the T-tubule invaginations.

Actually, the sarcolemma depolarization stimulates the voltage-gated calcium channel located in the T-tubule to release free calcium ions (Bers et al., 1998; Tanabe et al., 1990). This calcium channel found in the sarcolemrna is named the dihydropyridine receptor (DHPR) (Tortora et al., 1993). DHPR activates the calcium channel in the sarcoplasmic reticulum, referred to as the Ryanodine receptor (RyR), to open and release free calcium into the sarcomere (Tanabe et al., 1990 ; Mygland et al., 1992; Bers et al., 1998; Mouton et al., 2001; Lamb 2000).

The sarcoplasmic reticulum (SR) is a fluid-filled tubular structure whose main purpose is to store calcium when the muscle is in a state of rest. As the SR releases calcium into the sarcoplasm the calcium surrounds the thin and the thick filaments resulting in a muscle contraction. Calcium exits the SR via calcium release channels or RyR. Transverse tubules often called T-tubules are infoldings of the sarcolernma that take on a tubular shape. The function of the T-tubule is an open tunnel to fill with

extracellular fluid, containing a relatively high calcium concentration.

In order for muscle contraction to occur, energy is also needed in the form of ATP. The ATP binds to ATP receptors located on the myosin cross bridges. ATP is split, this occurs on a certain region of the myosin cross bridge acting as an ATPase. The energy is transferred from the ATP molecule to the myosin cross bridge to stimulate the cross bridge. Step one is the activation of the myosin cross bridge to bind to the myosin binding sites on the actin molecule. As the calcium concentration increases the tropomyosin slides away from the myosin binding sites on the actin molecule. Step two is the binding of the myosin cross bridge to the actin resulting in a conformational alteration to produce the contraction. As the myosin cross bridges slide toward the middle of the sarcomere to the H zone they release ADP. After the contraction the ATP binds to the ATP binding sites. When the ATP binds, the myosin cross bridge is released by the actin. Step four is the ATP molecule splitting to allow the myosin cross bridge to return to its normal conformation. Now, the myosin cross bridge can bind another actin molecule on the thin filament.

Initiation of relaxation depends on the degradation of ACh. Acetylcholinesterase (AChE) located in the synaptic cleft degrades ACh. In the absence of ACh the action potential is quelled and the calcium release

channels are closed. Calcium is pumped back into the SR resulting in a decrease in sarcoplasm calcium. When the calcium concentration decreases the tropomyosin-tropnin complex move over the myosin binding sites preventing interaction of actin and myosin.

The term myopathy is a general word used to describe any muscle disease. The classification for muscle myopathies is usually by the cause of the disease. Idiopathic muscle myopathy is a general term used when there is not a clinical reason for the behavior or dysfunction of the muscle. An uncommon example of a myopathy is an inflammatory myopathy. Criteria for classification as an inflammatory myopathy includes the presence of inflammatory cells, necrosis and phagocytosis of muscle fibers, fibers that are atrophic and regenerating simultaneously, and finally fibrosis. Some examples of inflammatory myopathies are; inclusion body myositis, polymyositis and dermatomyositis. Another classification of muscle myopathy is acquired autoimmune myopathy which have an unknown origin. For some unknown reason the body begins to develop antibodies to itself either the muscle fiber or the receptors. A few examples are; myasthenia gravis, which will be discussed in detail, and rhabdomyolysis a degradation of muscle fibers releasing myoglobin resulting in myoglobinuria. Also, there are genetic or inherited myopathies such as

Duchenne muscular dystrophy an X-linked disease causing muscle fibers to breakdown usually effecting the shoulder and pelvic girdles resulting in fibrosis. Another autoimmune myopathy is autoimmune rippling muscle disease.

In 1990 Dr. Carl Ansevin reported a case of autoimmune rippling muscle disease (AIRMD). At that time the patient displayed electrically silent rippling muscles without any other problems. In 1995 the same patient returned for treatment again. During this evaluation the patient presented with Myasthenia gravis (MG) as well as AIRMD. After the investagation of this patient's medical history and the absence of an inherited pathway it was concluded that the patient was suffering from an autoimmune condition.

Upon review of Dr. Ansevin's case study, there are several factors that allow us to conclude that the rippling muscle phenomenon is autoimmune in nature. First, the patient is the only one out of nine siblings to present with neuromuscular problems, weakness, RMD. This suggests that the rippling muscle disease is not of a genetic origin. An electromyography was negative for myotonia, thus concluding that nerve conduction studies are normal. This suggests that the RM phenomena is electrically silent. Secondly, the patient's condition improved when treated with pyridostigmine (mestinon) also known as (AchE) and prednisone. Prednisone is a steroid used to reduce the anti-inflammatory process that

occurs during an autoimmune condition. The fact that suppression of autoimmune reactivity improved our patient's condition also leads us to believe that the patient is suffering from an autoimmune condition. Thirdly, following treatment by plasmaphereses to remove antibodies the patient's condition improved. Finally, after the discovery of a thymoma and thymectomy the rippling muscle did not return and the MG symptoms dramatically improved (Ansevin et al., 1996). Since that time other AIRMD cases have been reported.

Rippling muscle contractions occur in the absence of motor unit action potentials (muap) (Ricker et al., 1989; So et al., 2001). It is believed that the propagation of contraction is induced by mechanical activation of calcium channels during percussion and stretching in patients with RM (Ansevin et al., 1996). There are several large muscle proteins that regulate calcium fluxes in the muscle fiber. As previously mentioned, a large muscle protein is the ryanodine receptor (RyR). RyR is a calcium release channel located in the sarcoplasmic reticulum. RyR is a transmembrane ion channel protein weighing 305 Kda residing in close contact to the T-tubular sarcolemma invaginations. Roughly half of the MG/Thymoma patients have IgG autoantibodies to RyR (Skeie et al., 2001). DHPR is a voltage dependent calcium channel located in the T-tubule membrane. DHPR consists of multimeric proteins weighing 170, 150, 52, 32 KDa with a grand total of 404 KDa (Mygland et al., 1994). It is suggested that the depolarization of the DHPR T-tubular membrane activates quick release of

calcium ions from the RyR into the sarcoplasm resulting in the rippling phenomena (Mygland et al., 1992). Also, the imrnunoreactivity pattern in patients presenting with RM/MG suggests a possible presence of autoantibodies to these calcium channels, according to their molecular weights (Walker et al., 1999).

Studies conducted in this laboratory include Western Blot assays on the patient's serum from 1995 and 1998 along with serum from two different patients exhibiting RMD and MG. These studies demonstrated autoantibodies in patients with RMD that recognize high and intermediate molecular weight antigens in skeletal muscle. The hypothesis behind these autoantibodies is that they can bind to the stretch activated channels releasing calcium via an unknown calcium channel with a high molecular weight or hypersensitivity to the dihydropyridine receptor that stimulates the ryanodine receptor to release calcium resulting in the rippling muscle phenomena. The autoantibodies were absent in patients presenting with MG only. The autoantibodies were present in patients presenting with MG, thymoma and rippling muscle. Preliminary data suggested immunoreactivity in the form of striational banding patterns.

Rippling Muscle

Rippling muscle disease is a rare, heterogenetic, autosomal dominant disease. This disease was first described by Torbergsen et. al in 1975 during an examination of a family with a disease similar to myotonia congenita.

Rippling muscle disease manifests as muscle weakness and stiffness after resting, a collection of mounding muscle movements after stretch or percussion. The muscle movements resemble a wave rolling across the entire muscle. These muscle contractions are post-synaptic occuring in the absence of a motor unit action potential (electrically silent) (Ricker et al., 1989; Kosmorsky et al., 1995; Ansevin et al., 1996). The genes responsible for causing rippling muscle disease (RMD) reside on the long arm of chromosome one and three (Vorgerd et al., 2001; Stephan et al., 1999).

There is a peculiar similarity between mechanosensitive channels (MSCs) and RMD. This similarity is muscle response upon stretch or percussion. Muscle responses are not propagated by neural sources and the ripple phenomena is electrically silent. It is suggested that the stretch activated wave-like contraction of skeletal muscle is a result of mechanosensitive calcium channels (Mygland et al., 1992). Due to these similarities, it is hypothesized that autoimmune rippling muscle patients generate autoantibodies to MSCs or stretch activated channels (SACs) resulting in a voltage change across the sarcolemma. This depolarization stimulates DHPR to release calcium to activate the RyR to release calcium.

MSCs were discovered in chick skeletal muscle by Guchray (Guchray et al., 1984). There are two types of MSCs, one is stretch inactivated channels (SICs) and the other type is stretch activated (SACs). When

stretch or percussion occur the SACs open permitting calcium to surge into the cell. Normally SACs are closed to ion flow. SICs are normally open to calcium current, however, during stretch or percussion they close.

Mechanical stimuli and amphipaths or a compound consisting of a strongly polar and a strongly nonpolar group like a phospholipid, or other chemicals such as trinitrophenol or chlorpromazine, open SACs more rapidly (Sokabe et al., 1993) and may involve the cytoskeleton. The same study also speculates SACs activation by cytoskeletal structures and lipids (Sokabe et al., 1993). Activation and inactivation may result in damage to the surrounding tissues as indicated in antother study on *mdx* mice. This study suggested MSCs regulation may cause pathophysiological calcium release (Franco-Obregon Jr. et al., 1994) due to neuromuscular disorders and muscular dystrophy. Integrins are proteins that attach extracellular matrix molecules to cytosketal proteins. Proteins of dystrophin-spectrin family are positioned under the membrane. Dystrophin has actin binding domains that can cause tension in the cytoskeleton stimulating MSCs (Hamill et al., 1995).

Another autoimmune disease linked to an exacerbation of autoantibody production, in patients with AIRMD, is myasthenia gravis (MG). MG is a neuromuscular disease distinguished by fluctuating weakness or exhaustion of skeletal muscle (Bartoccioni et al., 1980) because muscle contraction is partially obstructed, however contractility appears normal (Pagala et al., 1989). The most commonly affected muscles

are usually the throat and face muscles, especially the extraocular muscles surrounding the eye, but the neck, arm and leg muscles can be affected. Following muscle fatigue, slurred speech and problems with chewing and swallowing may occur.

Myasthenia gravis is an autoimmune neuromuscular disease resulting in an accumulation of antibodies that bind to the post-synaptic acetylcholine receptors on the motor end plate (Kimball et al., 1990). Due to the antibody accumulation, neuromuscular transmission is hindered, this is a result of incomplete coupling due to decreased neurotransmitter (acetylcholine) and the acetylcholine receptor binding. A study done by Bartoccioni in 1980 confirmed the presence of autoantibodies to the nicotinic acetylcholine receptors at the neuromuscular junction in myasthenia gravis (MG) patients. Muscle contraction is also reduced by the decreased quantity of normal acetylcholine receptors.

There are several other autoantibodies that exist in the MG patients. MG is exascebated by other immunological conditions like a thymoma, which results in B-cell proliferation and autoantibody levels to increase. A thymoma or hyperplasia is a lymphoepithelial tumor in the thymus gland that multiplies at a slow rate. An average of about 15% of MG patients exhibit a thymoma (Williams et al., 1986; Skeie et al., 1996). MG and thymoma patients have autoantibodies recognizing different intracellular muscle fiber proteins in the striations of the skeletal muscle myofibrils or A-bands, I-bands and A and I-bands located in the Z-line(Vetters 1967;

Williams et al., 1986). The striational autoantibodies are detected in 80-90% of patients with MG and thymoma. Other autoantibodies detected in MG and thymoma patients are to the skeletal muscle fiber proteins like myosin, actin, titin, alpha-actinin, tropomyosin (Skeie et at., 1996; Ohta et al., 1990; Pagala et al., 1990) and IgG autoantibodies to myosin and actin (Ohta et al., 1990). The last autoantibody found in patients with MG and thymoma occurs in about half the patients and is called the ryanodine receptor antibody. The titer of ryanodine receptor antibodies is directly proportional to the severity of the MG disease (Mygland et al., 1994).

Recent studies have shown a number of proteins associated with the dystroglycan complex resulting in muscle cell damage. A dystrophin deficiency leads to a loss of function in all the dystrophin-associated proteins leading to necrosis in Duchenne muscular dystrophy (DMD) (Ohlendieck et al., 1993). Dystrophin is a cytoskeletal membrane protein that interacts with extracellular and transmembrane glycoproteins, dystroglycan, laminin and actin, these proteins are dystrophin-associated proteins.

Another dystrophin-associated muscle problem is related to caveolin-3 a muscle specific gene. It interacts with the dystrophin-glycoprotein and it is localized to the sarcolemma. Caveolin-3 in conjunction with β-dystroglycan can control dystrophin localization to the muscle cell plasma membrane. Laminin-2 is linked to dystrophin by a-dystroglycan. β-

dominant and heterogenetic disease. The de novo CAV-3 patients have a decreased expression of a-dystroglycan and a reduced CAV-3 sarcolemrna distribution in the muscle fibers.

As if genetic mutations and protein deficiencies are not enough to cause muscular damage, free radicals can also cause muscular damage. One such free radical recently studied is nitric oxide (NO). NO is a free radical with a rapid half-life, it is also a nonadrenergic-noncholinergic transmitter. Nitric oxide synthase (NOS) is a calcium dependent enzyme that produces NO from L-arginine. Skeletal muscle manufactures NO using neuronal type NO synthase (nNOS) in the sarcolemma. The N-terminal domain of nNOS consists of a GLGF motif that associates with dystrophin. Dystrophin is responsible for the signaling enzyme in the muscle plasma membrane. A dysfunction in the signaling enzyme is thought to cause Duchenne Muscular Dystrophy (DMD) (Brenman et al., 1995).

There are also inducible calcium independent NOS (type II or iNOS) in cells during immunological processes like rheumatoid arthritis. It is believed that iNOS is accountable for cellular damage in immunological diseases. Calmodulin controls transcription of the NOS proteins (Bredt et al., 1990). An upsurge in cellular calcium may be due to NO synthesis. It is suggested that acetylcholine receptors (AChR) are clustered together by

agrin. The agrin also binds to dystrophin. An extracellular glycoprotein called a-dystroglycan connects with dystrophin (Campanelli et al., 1994). AChR clustering complex is comprised of AChR, agrin, dystrophin and α-dystroglycan. This complex is also known as the dystrophin complex. Damage to the dystrophin complex is thought to be accountable for muscular dystrophy (Campbell et. al. 1995). Cells that do not contain dystrophin accrue nNOS in the cytosol instead of the sarcolemma. Deviation in NO metabolism or NO reactions with superoxide will cause tissue damage in neurodegenerative and autoimmune diseases (Brenman et al., 1995).

Specific Aims

The purpose of my project was to determine the subcellular distributors of RMD autoantigens in skeletal muscle cells to determine the distributional relationship between RMD antigens, DHPR (T-tubules) and Ryanodine receptors (sarcoplasmic reticulum). Any correlation between the distribution of RMD antigens and DHPR or RyR suggests a possible functional relationship. This supports the results of the western blots performed by my colleagues Tom Watkins and Stacy Raab indicationg a T-tubule localization of RMD antigens. Distribution was determined by Immunofluorescence microscopy using antibodies from various types of MG patients.

Another specific aim was to determine if there were any cross-reactive antigens present in a mouse myocyte cell model system. The cell line used was (C2C12).

Experimental Design

The experiments conducted were constructed to establish the differences in immunoreactivity between the sera from a sample of diverse donor patients. The sample of patient sera consisted of three main patients. One of the main patient's sera was from a patient diagnosed with RM/MG/Thymoma. The two other patient's sera were from patients diagnosed with RM/MG/no thymoma.

This experiment was designed to identify the localization of antigens associated with autoimmune rippling muscles, and the patients with RM also have MG or MG and thymoma. The controls used have to distinguish RM irnmunoreactivity from MG immunoreactivity and thymoma irnmunoreactivity. One of the control patient's sera was from a patient diagnosed with MG. Antoher control was from a patient diagnosed with MG/Thymoma. Normal background irnrnunoreactivity occurring due to secondary antibody noise or immunoglobulins present in normal sera also required attention. In order to account for background immunoreactivity an asymptomatic patient's sera was used.

Commercially available antibodies were also required. Sigma anti-AChR, anti-DHPR and anti-RyR were used. The exact pattern of these antibodies needed to be documented to compare to the results of paatient's sera. Anti-AchR was expected to appear all over the sarcolemma. Anti-DHPR and anti-RyR was expected to appear as a periodic dot pattern at the skeletal muscle triad.

The sera from these patients and the controls were used as the primary antibody to identify the cellular localization of antigens in human skeletal muscle. The purpose of the secondary antibody was to serve as a fluorescent marker to recognize the location of the primary antibody.

Materials & Methods

C2C12 Cell Culture- First the cells were placed in a flask 20 % FBS. This media ensured the proper growth and division of the cells. When the cells reached a confluent state they were trypsinized and placed on cover slips. The cells on the cover slips were continuously monitored for the appropriate time to switch the media to low FBS. The low FBS induced the cells to differentiate. When the cells were differentiated they developed myotubes which displayed t-tubules, sarcomeres and other ultra structural characteristics of muscle fibers.

C2C12 Cell Preparation for **Immunofluorescence-First** the cells were fixed in a 2 % formaldehyde solution for 30 minutes. Next, they were rinsed

with TBS and placed in a 5 % blocking buffer solution with 250 µl Triton-X for 1 hour. Then rinsed with TBS 3 times for 10 minutes each. Rinsed cells were placed in 20 µl of primary antibody and 10 ml of 1 % blocking buffer, incubated for 1 hour at room temperature with gentle agitation. The primary antibodies used in this experiment were sera from the RM patients. The controls consisted of commercial primary antibodies like DHPR, AChR, RyR, and dystrophin. Next, rinsed cells with TBS 3 times for 10 minutes each. Added 20 µl of secondary antibody (goat anti human FITC) and 10 ml of 1% blocking buffer. Then rinsed the cells with TBS 3 times for 10 minutes each. An antioxidant was used, 5 % N-propyl gallate in glycerol, then a cover slip placed over the antioxidant and sealed with clear nail polish. The cells were properly prepared to be viewed under the microscope.

The cell preparation for the normal human skeletal muscle was the same as the C2C12 cell line except, the muscle tissue came from a fresh muscle biopsy, not a culture. Dr. Carl Ansevin provided the muscle tissue. Then muscle tissue was frozen and cut on a Leica CM 1800 cryostat microcryotome at –19 degrees Celsius by Keytam Awad at NEOUCOM. The thickness of the longitudinal fresh frozen muscle section was 10 um thick. Each experiment was performed the same way except the primary antibody was different and the corresponding secondary antibody differs depending on the primary antibody. Due to the fact that the C2C12 cell line could not be grown to differentiate in vitro the project was switched to

human skeletal muscle.

Fluorescent Microscopy-Fluorescence is described as light absorbed at one wavelength and emitted at a different wavelength. Autofluorescence, also referred to as primary fluorescence, occurs when the object can fluoresce in its natural state. Secondary fluorescence is when a chemical is used in order to fluoresce the object being viewed. The Autofluorescence was monitored by controls consisting of sera from MG/Thymoma and MG patients, commercial controls like anti-DHPR, anti-ACh, anti-Dystrophin and anti-RyR without the use of the secondary antibody FITC and with the use of FITC. Another control for autofluorescence was normal sera as the primary antibody and FITC as the secondary fluorescent antibody. All samples were viewed and photographed under the 100X objective with oil immersion for an exposure time of two minutes.

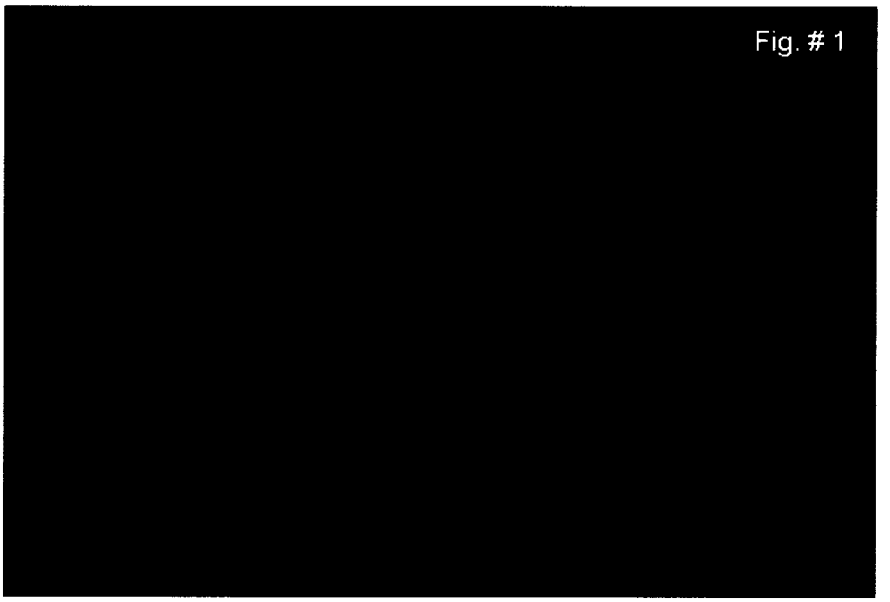

Figure 1 Background staining without an observable pattern
This image is a section of normal human skeletal muscle viewed at a magnification of 1000X and probed with primary antibody (serum) from a normal control at a concentration of 1: 500. The secondary antibody used is goat α l FITC [1: 1000]. There is an absence of a specific immunoreactivity pattern consistent with a normal control non flurrid.

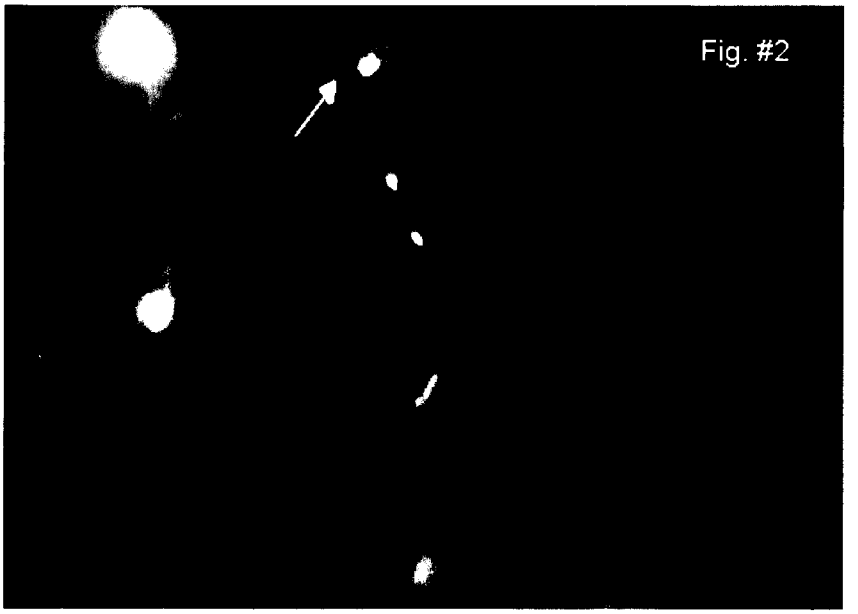

Figure 2 Distinct striational banding pattern with sera from MG/Thymoma-patient
This figure is a micrograph reporting the results of immunofluorescent analysis of a MG/Thymoma patient's sera photographed at a total magnification of 3640X. Sera was applied to a thin section of human skeletal muscle at a 1:1000 concentration. FITC labeled antibody was applied at a concentration of 1:1000. **A** visible striational pattern is apparent along the margin of the muscle cells (see arrow) as well as immunoreactivity along the periphery of the sarcolemma.

Figure 3 Striational banding pattern and periphery staining with MG/RM/Thymoma patient's sera

This is an image of a fresh frozen section of normal human skeletal muscle labeled with primary antibody from a patient with MG/Thymoma and active RM at a concentration of 1:500. The secondary antibody used is goat *a* 1 FITC at a concentration of 1:1000 and photographed at a total magnification of 4350X. The immunofluorescent micrograph shows an observable striational banding pattern (see arrow number 2). Also, there is a staining pattern on the periphery of the sarcoplasm (see arrow number 1).

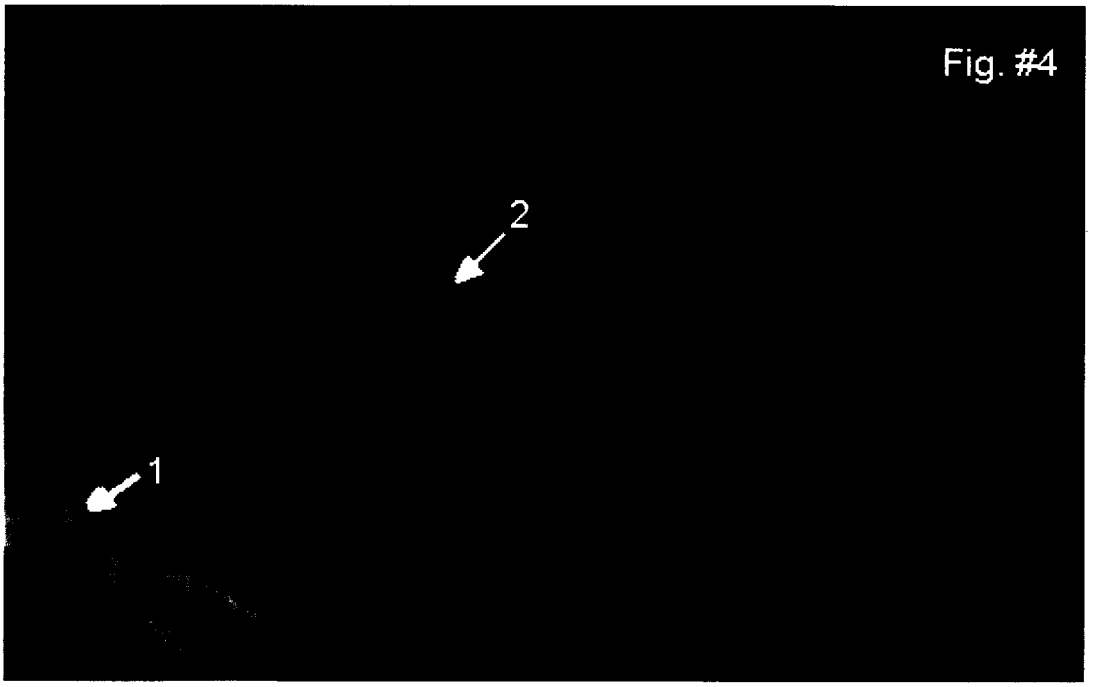

Figure 4 Striational banding pattern and periphery staining with MG/RM/Thymoma patient's sera

This is an image of a thinly sliced fresh frozen section of normal human skeletal muscle probed with sera from a patient with MG/RM/Thymoma as the primary antibody at a concentration of 1:1000. The secondary antibody used is goat *a* l FITC at [1:2000] and photographed at a total magnification of 4480X. The immunoreactivity shown is on the periphery (see arrow number 1) as well as a striational banding pattern (see arrow number 2).

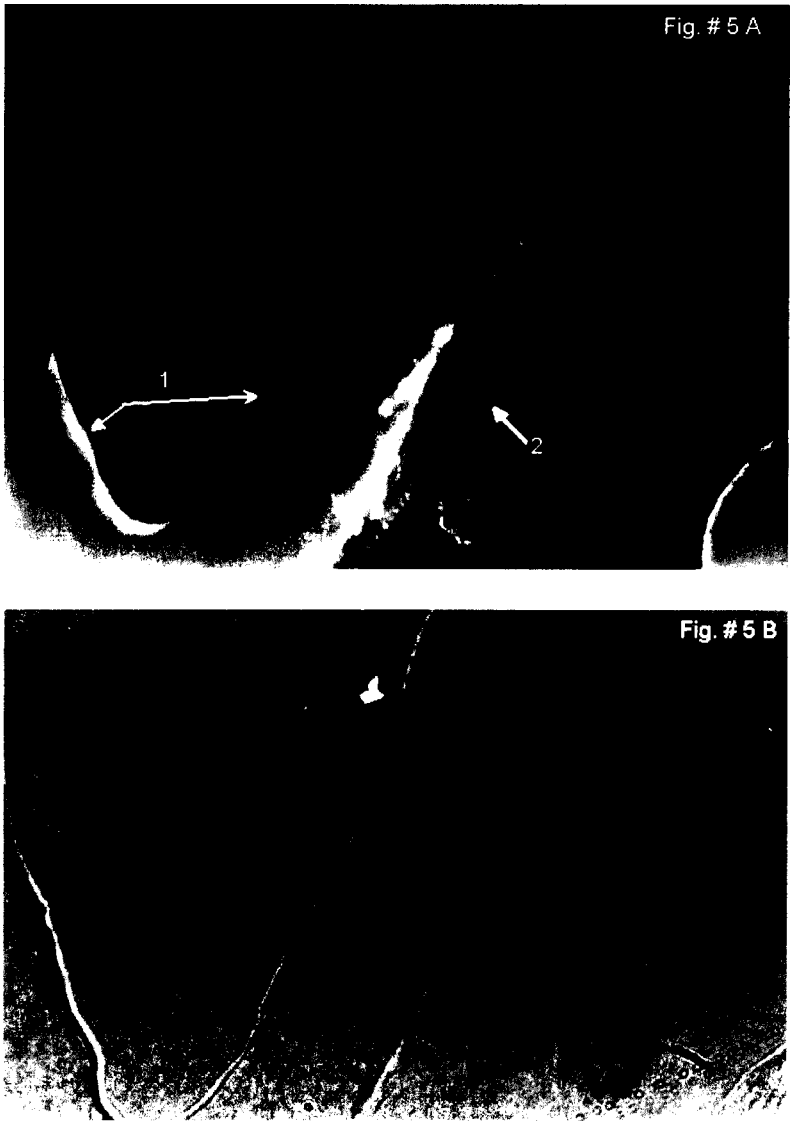

Figure 5 Striational banding pattern and periphery staining using sera from a patient with MG/RM/ no thymoma

The upper micrograph is a fluorescent micrograph and the lower micrograph is a phase contrast micrograph, both photographed at a total magnification of 3520X. These figures are from thinly sliced fresh frozen section of normal human skeletal muscle labeled with sera from a patient with MG/RM/no thymoma as the primary antibody at a concentration of 1:250. The secondary antibody used is goat a l FITC at [1:1000] both micrographs were taken at a magnification of 1000X. The immunoreactivity is observed as a striational banding pattern (see arrow number 2) as well as an observable immunoreactivity along the periphery (see arrow number 1) of the sarcoplasm.

Figure 6 Striational banding pattern in conjunction with periphery staining using sera from a patient with MG/RM/ no thymoma.

This image is a thinly sliced fresh frozen section of normal human skeletal muscle probed with primary antibody at a concentration of 1:500 from the sera of a different patient with MG/RM/no thymoma. The secondary antibody is goat a l FITC at [1:2000]. The upper image is a micrograph using a fluorescent microscope and the lower image is a micrograph using a phase contrast microscope both taken a total magnification of 3640X. The observable immunoreactivity pattern presents as a striational banding pattern (see arrow # 2) as well as along the periphery (see arrow # 1) of the sarcoplasm.

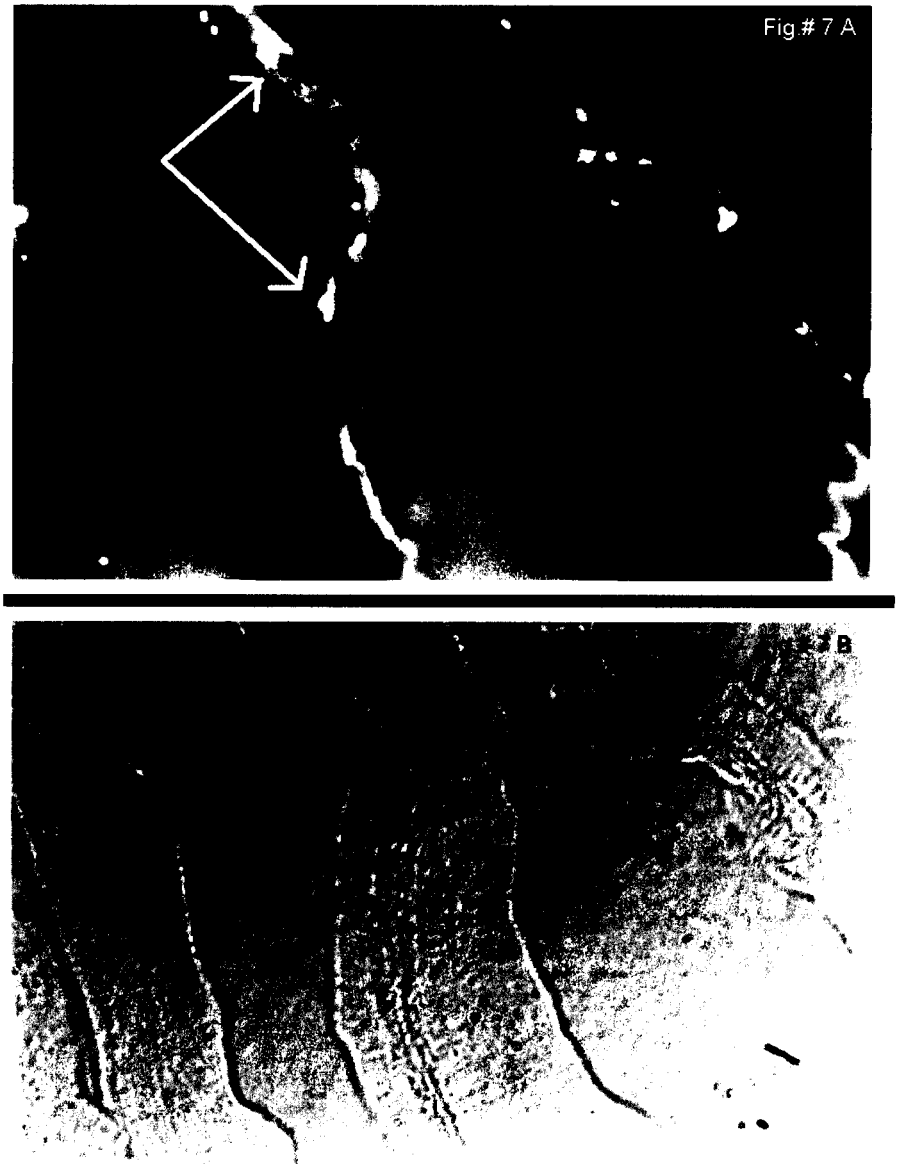

Figure 7 Intense specific binding using anti-dystrophin at a total magnification of 3680X.

The upper micrograph is an image using a fluorescent microscope and the lower one is from a phase contrast microscope. These figures are thinly sliced fresh frozen section of normal human skeletal muscle labeled with dystrophin at a concentration of 1:500 as the primary antibody. The secondary antibody is GXM FITC at [1:1000]. The observable immunoreactivity shows intense specific binding on the sarcolemma (see arrow).

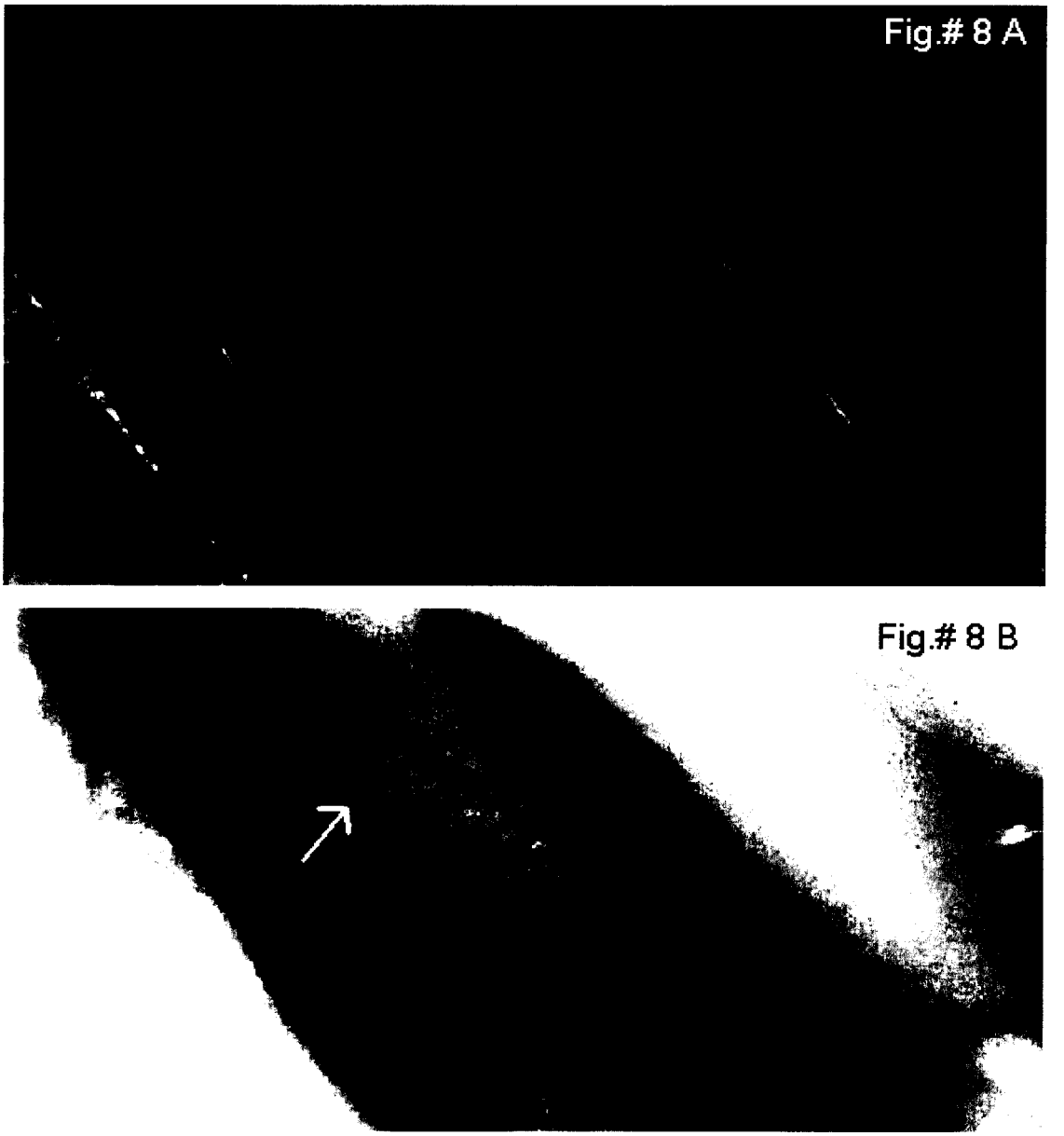

Figure 8 Striational banding pattern of DHPR distribution
These micrographs are thinly sliced fresh frozen section of normal human skeletal muscle probed with anti-DHPR as the primary antibody at a concentration of 1:1000. The secondary antibody used is GXM FITC at a concentration of 1:500. The upper image is a micrograph using a phase contrast microscope and the lower image is a micrograph using a fluorescent microscope, both taken at a total magnification of 4500X. The observed immunoreactivity is an intense, more pronounced striational banding pattern (see arrow).

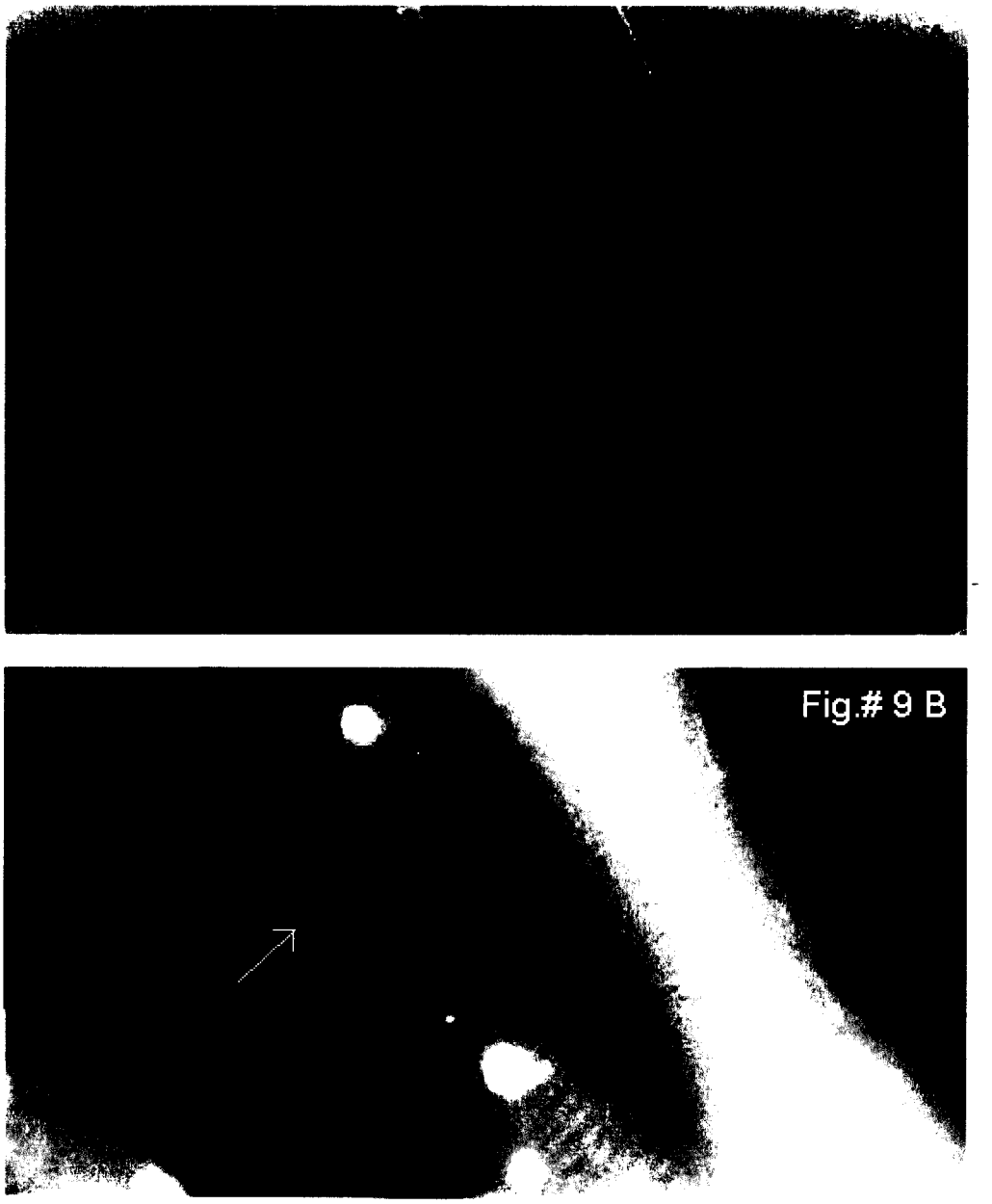

Figure 9 Striational banding pattern of RyR distribution
Both images are thinly sliced fresh frozen section of normal human skeletal muscle labeled with anti-RyR as the primary antibody at a concentration of 1:500. GXM FITC is the secondary antibody used at a concentration of 1:1000. The upper micrograph is an image taken from a phase contrast microscope and the lower micrograph is an image taken from a fluorescent microscope both taken at a total magnification of 3960X. The observable immunoreactivity is an intense, more pronounced striational banding pattern (see arrow).

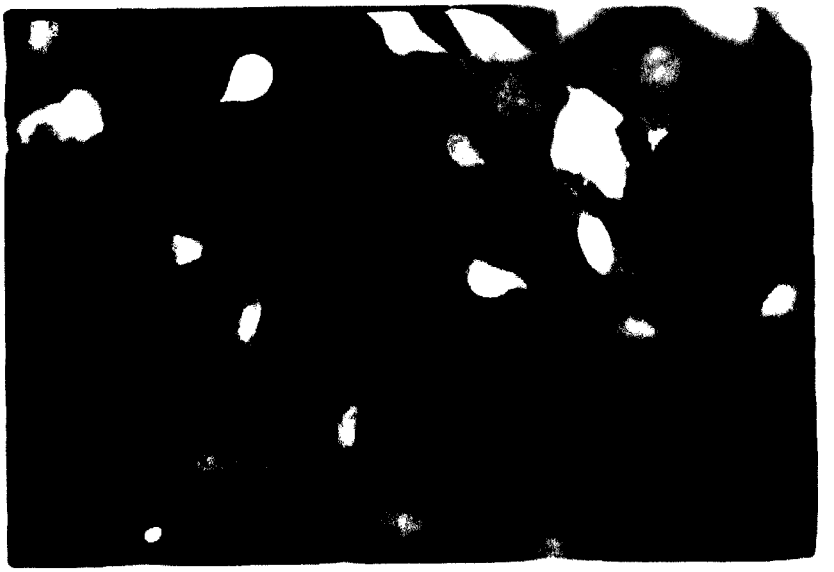

Figure 10 Acetylcholine Receptor Antibody with C2C12 cells
This is a figure of cultured C2C12 contractile muscle cell line, at the myoblast stage, probed with mouse *a* human AChR as the primary antibody at a concentration of 1:500. The secondary antibody used is GXM FITC at a concentration of 1:1000. Figure 10 shows an intense immunofluorescent staining through out the entire cell.

Figure 11 DHPR antibody with C2C12 cells
Figure 11 is a fluorescent micrograph of culture C2C12 contractile muscle cells, at the myoblast stage, labeled with primary antibody anti-DHPR at a concentration of 1:500. The secondary antibody is GXM FITC at a concentration of 1:1000. This figure illustrates a diffuse immunoreactivity through out the entire cell.

TABLE 1 Fluorescent striational band width

FIGURE NUMBER	PATIENT DESCRIPTION	TOTAL MAGNIFICATION OF PRINT	SCALE OF PRINT um	AVERAGE INTRA-STRIATIONAL BAND DISTANCE um	STANDARD DEVIATION
2	MG/Thymoma	3640	0.27	0.36	0.16
3	MG/RM/ THYMOMA	4350	0.23	0.42	0.07
4	MG/RM/ THYMOMA	4480	0.22	0.41	0.07
5	MGRM no thymoma	3520	0.28	0.47	0.17
6	MG/RM no thymoma	3640	0.27	0.60	0.08
7	ANTI-DYSTROPHIN	3680	0.27	NA	NA
8	ANTI-DHPR	4500	0.22	0.67	0.0
9	ANTI-RyR	3960	0.25	0.67	0.15

TABLE 2 Repetitive striational band distance

FIGURE NUMBER	**PATIENT DESCRIPTION**	**TOTAL MAGNIFICATION OF PRINT**	**SCALE OF PRINT um**	**REPETITIVE STRIATIONAL BAND DISTANCE um**	**STANDARD DEVIATON**
2	MG/possible thymoma	3640	0.27	0.45	0.20
3	MG/RM/ THYMOMA	4350	0.23	0.51	0.19
4	MG/RM THYMOMA	4480	0.22	0.56	0.0
5	MG/RM no thymoma	3520	0.28	0.62	0.08
6	MG/RM no thymoma	3640	0.27	0.60	0.08
7	ANTI-DYSTROPHIN	3680	0.27	NA	NA
8	ANTI-DHPR	4500	0.22	0.67	0.0
9	ANTI-RyR	3960	0.25	0.72	0.08

TABLE 3 Distance between the fluorescent bands

FIGURE NUMBER	**PATIENT DESCRIPTION**	**TOTAL MAGNIFICATION OF PRINT**	**SCALE OF PRINT um**	**DISTANCE BETWEEN BANDS um**	**STANDARD DEVIATON**
2	MG/possible thymoma	3640	0.27	0.21	0.20
3	MG/RM/ THYMOMA	4350	0.23	0.23	0.0
4	MG/RM THYMOMA	4480	0.22	0.22	0.0
5	MG/RM no thymoma	3520	0.28	0.20	0.08
6	MG/RM no thymoma	3640	0.27	0.27	0.0
7	ANTI-DYSTROPHIN	3680	0.27	NA	NA
8	ANTI-DHPR	4500	0.22	0.22	0.0
9	ANTI-RyR	3960	0.25	0.25	0.0

TABLE 4 RESULTS SUMMARY

FIGURE NUMBER	DHPR	RyR	AChR Dystrophin	I-band	A-band
2	–	–	+	+	–
3	–		+	+	–
4	–	–	+	+	–
5	–	–	+	+	–
6	+	+	+	–	–

RESULTS

A patient with no clinical symptoms or diagnosis of any type of neuromuscular disease was used as the primary antibody for a negative control (Figure 1). The negative control showed that the fluorescence was present however, it was nonspecific and very much less intense compared to the other figures. This data demonstrated an absence of any type of immunofluorescent striational banding pattern suggesting background staining. The purpose of this control was to access background fluorescence patterns.

The average intra-band as inter-striational distance is the width of the fluorescent striational band (Table 1). The repetitive striational band distance is the periodicity of the fluorescent bands (Table 2). Table 3 is the distance between the bands or the dark area that is not fluorescent.

Imrnunoreactivity displayed a striational banding pattern as well as at the periphery of the sarcolemma; however, not as intense as the rest of the figures, but definitely more intense than the asymptomatic control. Serum from a patient with MG and a Thymoma was used as a source of primary antibody as the MG/Thymoma control (Figure 2). The average intra-band as inter-striational distance or average fluorescent band width measured 0.36 μm (Table 1). Fluorescent striational bands occurred about every 0.45

μm (Table 2). The distance between the striational bands or the dark area measured 0.21 μm (Table 3). The sum of all the structures of a sarcomere should be between 2-2.4 pm, the length of a sarcomere (Tortora et. al., 1993). Sarcomere length varies depending upon whether or not a sarcomere is relaxed (a longer length) or contracted (a shorter length) (Tortora et. al., 1993).

A student T-test calculation was used to compare the data between each patient and the DHPR and the RyR controls. Thus, the student T-test rejected the null hypothesis that the fluorescent striational band width of the MG/Tymoma patient (Figure 2) correlates with the cellular localization of DHPR (Figure 8) or RyR (Figure 9) controls. This suggested that the striational banding pattern of the MG/Thymoma patient's sera was not consistent with the cellular localization of DHPR or RyR.

However, another student T-test failed to reject the null hypothesis that compared the immunofluorescent striational banding pattern of the MG/Thymoma patient (Figure 2) to the cellular localization of the I-band within the sarcomere. The width of the I-band in a sarcomere measured 0.44 μm in an atlas of frog skeletal muscle (Porter et. al., 1973). The measured I-band width was compared to the width of the fluorescent striations measured from figure two. This demonstrated that the width of

the patient's striational banding pattern matched the width of an I-band in a sarcomere.

Sera obtained from RM/MG/Thymoma patient showed a striational banding pattern (Figures *3* and 4). This patient has active RM/MG/Thymoma. In figure three the concentration of primary antibody was 1:500 and the secondary antibody concentration was 1:1000. In figure four the concentration of the primary antibody was 1:1000 and the concentration of the secondary antibody four was 1:2000. There is very little variation observed in the staining pattern with the same sera at different concentrations.

Both figure three and four displayed an intense and distinct striational banding pattern as well as immunoreactivity on the margin of the sarcolemma. The average intra-band as inter-striational distance or width of the fluorescent striational bands in figure three was 0.42 μm and figure four was 0.41 μm (Table 1). Also, the fluorescent striational bands occurred about every 0.51 μm (Figure 3) and for figure 4 about every 0.56 μm (Table 2). The distance between the striational bands or the dark area for figure 3 was 0.23 μm and for figure 4 was 0.22 μm (Table 3). Data from the measurements of figure three and figure four show accuracy and precision.

A student T-test calculation was used to compare the data of the RM/MG/Thymoma patient, used in figures three and four, to the DHPR and RyR controls. This showed that the RM/MG/Thymoma pattern is in fact different than either the DHPR or RyR pattern. The student T-test rejected the null hypothesis that the width of the fluorescent striations from the sera of the RM/MG/Thymoma patient correlate with the cellular localization of the DHPR or the RyR controls.

A second student T-test was calculated to compare the data measurements obtained from the results of figure three and four. The student T-test failed to reject the null hypothesis that the width of the fluorescent striations for the RM/MG/Thymoma patient's sera was the same as the width of the I-band within the sarcomere based on the I-band width measured 0.44 μm from an atlas of frog skeletal muscle (Porter et. al., 1973). Our failure to reject the null hypothesis suggested that the width of the patient's striational banding pattern was similar to the width of an I-band in a sarcomere.

An observable striational banding pattern and immunoreactivity on the periphery of the sarcolemma was displayed (Figure 5). The average intra-band as an inter-striational distance or width of the fluorescent striations measured 0.47 μm (Table 1) and the fluorescent striational bands

occurred about every 0.62 μm (Table 2). Also, the distance between bands or the dark area between the fluorescent bands measured 0.20 μm (Table **3).** This figure used sera from a patient with MG/RM/no thymoma as the source of primary antibody.

The student T-test calculation was performed to compare the data measured for the fluorescent striational band width obtained from the serum of a patient with MG/RM/no thymoma (Figure *5)* to the fluorescent striational band width of the DHPR and the RyR controls. This calculation showed that the MG/RM/no thymoma pattern was different from either DHPR or RyR pattern. Results of the calculation rejected the null hypothesis that the width of the striations of the MG/RM/no thymoma patient is equal to the cellular localization of the RyR or DHPR controls based on the distance between T-tubule structures. This illustrated that the striational banding pattern of the MG/RM/no thymoma patient's sera was not connected to the cellular localization of the RyR or DHPR.

Another student T-test calculation failed to reject the null hypothesis comparing the width of the fluorescent striational banding pattern observed from the sera of a patient with MG/RM/no thymoma (Figure *5)* to the cellular localization of the I-band within a sarcomere. The I-band width was measured from an atlas with frog skeletal muscle (Porter et. al., 1973).

The width of the I-band measured 0.44 pm. Therefore, the width of the patient's fluorescent striational banding pattern correlates with the width of the I-band in a sarcomere.

Serum from a different patient with MG/RM/no thymoma displayed irnrnunoreactivity on the margin of the sarcolemma and a concise striational banding pattern (Figure 6). The average width of the fluorescent band was 0.60 μm (Table 1). Repetitive striational banding pattern distance or fluorescent striations appeared about every 0.60 μm (Table 2) and the distance between the bands, the dark area, was 0.27 μm (Table 3). The results of figure six are slightly different from figure five. A student T-test calculation was performed to compare the width of the fluorescent striational bands of the MG/RM/no thymoma patient to the DHPR and RyR controls. Results of the calculation failed to reject the null hypothesis that the width of the striations of the MG/RM/no thymoma patient were the same as the expected widths of T-tubules based on the cellular localization of RyR and DHPR controls. This suggested that the striational banding pattern of the MG/RM/no thymoma patient's sera was similar to the cellular localization of the RyR and DHPR.

A student T-test rejected the null hypothesis that compared the striations of the MG/RM/no thymoma patient to the cellular localization of

the I-band within the sarcomere. The width of the I-band was measured at 0.44 μm from an atlas of frog skeletal muscle (Porter et. al., 1973). The student T-test showed that the width of the patient's striational banding pattern does not correspond to the width of the I-band in a sarcomere.

Anti-dystrophin labeling resulted in an intense irregular fluorescent pattern that is localized at the periphery of the cells (Figure 7). This suggested a sarcolamina localization or plasma membrane. All of the patients except for the asymptomatic control exhibited immunoreactivity at the periphery of the sarcolemma. These patients may possibly contain an autoantibody to dystrophin.

An intense more concise striational banding pattern was displayed by anti-DHPR (Figure 8) that may correspond to T-tubules. The observed immunoreactivity was a striational banding pattern. There was no detectable staining on the periphery of the sarcolemma. This figure was a control to determine the type of immunoreactivity pattern and compare it to the patient's imrnunoreactivity pattern. The width of the fluorescent striational bands measured 0.67 μm (Table 1). The repetitive striational band distance or the fluorescent bands occurred about every 0.67 μm (Table 2). The distance between the bands, the dark area, was 0.22 μm (Table 3).

Likewise, the RyR control displayed immunoreactivity with an intense and more concise striational banding pattern (Figure 9) that may correspond to the T-tubules. The immunoreactivity displayed a fluorescent striational banding pattern without immunoreactivity on the margin of the cells. Figure 9 was a control to determine the type of imrnunoreactivity pattern and compared it to the patient's immunoreactivity pattern. The width of the fluorescent striations measured 0.67 μm (Table I). The fluorescent bands occurred about every 0.72 μm (Table 2) and the distance between the fluorescent bands measured 0.25 μm (Table **3).**

C2C12 cells were probed with the antibodies to determine antigen distribution in myoblast. Acetlycholine was distributed in a diffuse pattern in the myoblasts (Figure 10). In mature muscle cells AChR should be localized to the motor end plates. These cells are mostly myoblasts since they are circular and mononuclear. Since these cells were not differentiated there are few myotubules observed.

DHPR was distributed in an intense nuclear staining and a diffuse plasma membrane staining was observed (Figure I I). Most of the cells are myoblasts because they are circular and mononuclear. There are very few myotubes observed.

The AChR used as a primary antibody was also a control (data not shown). AChRs are located on the motor end plate of the muscle sarcolemma. The anti-AchR control showed immunoreactivity throughout the sarcolemma. The micrograph displayed aggregations of intense irnrnunfluorescence without forming a discernable pattern. The MG patient's sera was also a control (data not shown). The micrograph showed fluorescence all over the plasma membrane of the muscle cell lacking a concise pattern. However, the immunofluorescence was not as intense as the AChR control. The purpose of an MG control was to identify the immunoreactivity pattern of a MG patient to compare it with the RM/MG/Thymoma. This control accounts for anti-AChR immunofluorescence.

DISCUSSION

The anti-AChR control displayed intense immunoreactivity along the periphery of the sarcolemma (data not shown). The MG patient control also exhibited immunoreactivity along the periphery of the sarcolemma (data not shown). However, the MG patient control's immunoreactivity was not as intense as the anti-AChR control's immunoreactivity. All of the sera from the patients displayed immunoreactivity along the margin of the sarcolemma. The immunoreactivity intensity was similar to the MG patient control, not the anti-AChR control. Since 85% of MG patient's sera contain autoantibodies to the AChR (Romi et al., 2000) these results were expected. It has been reported that autoantibodies to AChR obstruct neuromuscular transmission and contraction by complement-mediated sarcolemma damage (Lindstrom et al., 1988). If this is the case, the damaged sarcolemma would provide an entry for autoantibodies like RyR and titin.

The anti-RyR and anti-DHPR controls displayed a very similar cross striational banding pattern. These results were unexpected. The expected immunoreactivity pattern should have appeared as distinct, concentrated spots positioned as a double set of transverse lines consistent with sarcomeric spacing (Takekura et al., 1999). In the study conducted by Takekura et al., 1999, the immunofluorescent label controls used were alpha

1 DHPR and RyR1 on normal adult mouse muscle. The sum of the spots forms a cross striation pattern like the one observed in the results section for the DHPR and RyR controls. However, each spot was observed distinctly due to their use of higher magnification used by Takekura et al., 1999. Only one patient revealed a similar cross striation pattern like the anti-RyR and anti-DHPR controls. This suggests that the patient's sera possibly recognized an antigen of the human skeletal muscle with a cellular location similar to RyR or DHPR.

Another study reported that autoantibodies to RyR are produced in about 50-76% of patients with MG/Thymoma and late on set MG (Skeie et al., 2001). Usually once these patients produce antibodies to RyR the MG symptoms become more severe leading to weakness of respiratory muscles and respiratory failure (Skeie et al., 2001). It has been reported that 95% of MG patients with a cortical thymoma, form autoantibodies to titin and RyR and the primary autosensitization against RyR and titin antigens occurs inside the thymoma (Romi et al., 2002). Titin and RyR autoantibodies are usually of IgG subclass 1 or 3 which activate the complement cascade (Romi et. al., 2000). This suggests the muscle damage would increase due to the complement cascade.

Dystrophin is located in the I-band of a sarcornere. An expected result for the dystrophin control would be immunoreactivity along the sarcolemma. The dystrophin control displayed an intense immunoreactivity along the periphery of the sarcolemma. All of the patients displayed immunoreactivity along the periphery of the sarcolemma possibly suggesting autoantibodies to dystrophin in these patients.

The width of the A-band form an atlas of frog skeletal muscle is 1.9 μm (Porter et al., 1973) which does not correlate with any of the fluorescent striational band widths for patient sera or commercial antibody controls. This suggested that the cross striation pattern observed does not correspond to the A-band of the sarcomere. The width of the Z-line form an atlas of frog skeletal muscle is 0.072 μm (Porter et al., 1973) which does not agree with any of the fluorescent striational band widths for patient sera or commercial controls. This suggested that the cross striation pattern observed does not correspond to the width of the Z-line. However, the width of the I-band is 0.44 μm (Porter et al., 1973) which agrees with all of the fluorescent striational band widths from patient sera except for one of the patient's sera with MG/RM/no thymoma (refer to figure six). This suggested that the sera from these patients recognized an antigen in the human skeletal muscle located in the I-band. The cross striation pattern

appeared similar to the cross striation pattern from a study that used rat myocytes to demonstrate that the cross striation pattern of the I-band was finer than the A-band but similar to titin (Hein et al., 1994).

Previous experiments conducted in our laboratory included Western blots, immunoprecipitation and sub-cellular fractionation have indicated that the muscle proteins that are possibly involved with AIRMD are high molecular weight proteins. Western blots demonstrated immunoreactivity to a very high molecular weight protein between 400 kDa and 300 kDa. Also, immunoreactivity to lower molecular weight protein (200 kDa) and lower molecular weights were also present in the resolving gel. These autoantigens were present in the stacking gel (>400 kDa) with antibody detection, using serum from patients with RM, but these were not identified in the MG patient's sera, therefore, appear to be rippling muscle specific (Walker et al., 1999; Watkins Master thesis 1998). MG/ Thymoma patient's sera also displayed autoantibody immunoreactivity to very high molecular weight antigens in the stacking gel and high molecular weight antigens in the resolving gel (Walker et al., 1999; Watkins Master thesis 1998). Thymoma patients presented with a decreased irnmunoreactivity to these antigens. Controls included normal asymptomatic patient's sera as well as patients with MG only. MG patient's sera exhibited immunoreactivity at a

molecular weight range of 40-70 kDa, the secondary antibody also displayed immunoreactivity at this molecular weight (Watkins Master thesis 1998). Since the secondary antibody was used as a control, it is thought that this immunoreactivity is due to nonspecific binding due to the secondary antibody.

An anticipated immunoreactivity to the acetylcholine receptor subunits within this molecular weight range was expected since all the patients had MG. An intact acetylcholine receptor has a molecular weight of about 290 kDa (Changeux et. al. 1984) and was not present either. The nicotinic acetylcholine receptor consists of five subunits; 2 *a*, 1 β, ly, 1 6 and 1 ε that replaces 6 when it is junctional (Taylor et al., 1999). These subunits have molecular weights between 40-60 kDa (Taylor et al., 1999). There is immunoreactivity in the molecular weight range of 40-70 kDa suggesting the autoantibodies are recognizing a portion of nAChR subunit. It is a known fact that MG patients do have antibodies to acetylcholine receptors. Denaturation of patient's sera during SDS-PAGE may have disrupted the antigen-antibody binding site or freezing and thawing of patient's sera may have decreased antibody activity.

RM patient's sera exhibited recognition of very high molecular weight antigens in the stacking gel may perhaps recognize the calcium

release channel of the sarcoplasmic reticulum, the ryanodine receptor (RyR) because RyR has a molecular weight of 350 kDa. Recent cDNA library screening (DNA sequence) studies suggest recognition of titin antigens by RM patient's autoantibodies in the stacking gel. Also, a titin isoform maps to 1q42 region, the same location as a RM mutation (Personal communications with Thomas C. Watkins 2002). The high molecular weight antigen found in the resolving gel has not been identified to date.

The sub-cellular fractionations of rat skeletal muscle probed with sera from RM/MG patients showed autoantibody recognition of antigens in complex with DHPR (Walker et al., 1999; Watkins et al., 2001). Likewise, imrnunoprecipitation experiments done using sera from RM/MG patients show immunoreactivity to very high molecular weight antigens in their native state. This suggests autoantibody recognition to the T-tubule DHPR complex. An association between RyR in the sarcoplasmic reticulum and DHPR in the T-tubule membrane exists (Marx et. al. 1998).

The correlation of the previous results and the results from my immunofluorescent micrographs suggest that the very high molecular weight antigen (>400 kDa) in the stacking gel are recognized by autoantibodies from RM patients located in the T-tubule/ DHPR/ region of the skeletal muscle triad. High molecular weight antigens in the resolving

gel about 300 kDa are recognized by autoantibodies may be RyR located in the skeletal muscle triad. The striational banding pattern found in the serum of patients with RM or thymoma may correspond to the I-band region where the skeletal muscle triad is located. At the I-band there is titin, T-tubule1 DHPR, RyR, troponin, tropomyosin and dystrophin. DHPR is activated by the depolarization of the sarcolemrna to stimulate calcium release from the sarcoplasmic reticulum via RyR (Tanabe et al., 1990; Bers and Fill, 1998). Therefore, an autoantibody may bind to DHPR to stimulate RyR to activate the release of calcium. This is a possible theory to explain the stretch or percussion activation of autoimmune rippling muscle.

As previously mentioned the caveolin gene family is muscle specific and is also involved with the genetic form of RMD. Caveolin-3 is detected in the sarcolemma by a direct link with β-dystroglycan. β-dytroglycan is an integral membrane protein found in the dystrophin complex. PPXY motif in the C-terminus end of the β-dystroglycan interacts with the WW-like domain of caveolin-3. Dystrophin association to β-dystroglycan is competitively blocked by caveolin-3 (Sotgia et al., 2000). The caveolin-3 and β-dystroglycan interaction may control the dystrophin localization to the sarcolemma, maintaining and controlling sarcolemmal activity (Sotgia et al., 2000). A recent study identified caveolin-3 gene, on chromosome 3p25,

mutations responsible for autosomal dominant RMD (Vorgerd 2001). There are four caveolinopathies or missense mutations in the caveolin-3 gene (CAV-3) that pertain to RMD. A de novo mutation in CAV-3 is accountable for a fractional decrease of CAV-3 expression at the sarcolemma and a decrease of a-dystroglycan with normal nNOS levels. The missense mutation exchanges arginine for glutamine on the N-terminus end of the first exon of CAV-3. Caveolins impede nNOS and caveolin-3 directly associates with nNOS. Caveolinopathies have normal nNOS expression with increased inducibility of nNOS and increased calcium release which cause diseased muscle (Vorgerd et al., 2001). The speculation of a commonality shared between the RM, caveolinopathies, protein deficiencies and genetic mutations is that they all affect muscle. Muscle damage most likely occurs due to an increased calcium concentration causing more contractions than normal muscle.

However, the autoantibodies found in AIRMD patients do not localize to the same region as caveolin-3. Caveolin-3 is located on the surface of the sarcolemma surrounding the myocytes. My data indicated autoantibodies of AIRMD patients fluorescently localize in a cross striational banding pattern at the surface similar to the region where the skeletal muscle triad is found.

These recent studies have a peculiar common location to the I-band. Laminin-2 is linked to the extracellular matrix (Cohn et al., 2000). Dystroglycan consists of an a and β subunits, the a connects laminin-2 to the β-dystroglycan which is embedded into the sarcolemma (Cohn et al., 2000). β-dystroglycan is next to the sarcospan and sarcoglycan complex. Dystrophin is anchored by F-actin, in the Z-disc, and connects β-dystroglycan to syntrophins. Syntrophins are the site of nNOS and CAV-3. Dystrophin is located in the I-band region (Tortora et al., 1993). The striational banding pattern demonstrated in the fluorescent micrographs with patients that have AIRMD are similar to the I-band region. The I-band is also the site of the skeletal muscle triad (Tortora et al., 1993). This suggests the I-band is a recognition site for autoantibodies in RM patients.

It is a known fact that MG patients have autoantibodies to the nAChR that ultimately produce a neuromuscular dysfunction. It is also known that genetic mutations and protein deficiencies as well as free radicals can lead to muscular damage. It is possible that AIRMD patients synthesize autoantibodies that recognize proteins associated with the I-band. These autoantibodies may identify SACs allowing ion movement causing a depolarization of the myocyte. This allows calcium to enter via DHPR to

stimulate calcium release from RyR resulting in a stretch or percussion activated muscle ripple.

A possible mechanism for the wave-like contractions to occur in AIRMD would be due to the autoantibodies. There may possibly be autoantibodies to the DHPR that bind to it leading to a conforrnational change allowing for mechanically stimulating activation of the RyR to release calcium leading to wave-like ripples.

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