EFFECTS OF MUSCLE CRUSH INJURY ON THYMIC MYOID CELLS IN CONTROL AND *MDX* DYSTROPHIC MICE

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EFFECTS OF MUSCLE CRUSH INJURY ON THYMIC MYOID CELLS IN CONTROL AND MDX DYSTROPHIC MICE

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ABSTRACT

Muscle precursor cells (mpcs) used in muscle regeneration in adult muscle are satellite cells. An X-linked mutation in both the *mdx* mouse and Duchenne muscular dystrophy (DMD) patients produces an absence of dystrophin protein, important for membrane stability during contraction. DMD is fatal, but *mdx* mice don't die from dystrophy. *Mdx* mouse muscles experience cycles of damage and repair, making the model invaluable in the study of muscle regeneration. Thymic myoid cells have skeletal muscle characteristics, such as striations and expression of muscle-specific regulatory genes. Also, skeletal muscle can be grown from cells of the thymus. It was proposed then that myoid cells could potentially serve as another source of mpcs. *In situ* hybridisation, electron microscopy histochemistry, immunohistochemistry, and autoradiography were used to test the hypothesis that if myoid cells were mpcs for muscle regeneration, myoid cell density and distribution would differ in control and *mdx* mice after a crush injury to the right tibialis anterior. The appearance of myoid cells was comparable to previous reports. The troponin T-positive myoid cell population was significantly higher in control than in *mdx* thymus. Large myoid cells showed the greatest response to the muscle injury, which differed significantly (interaction effect, $p<0.0001$, ANOVA) between the 2 strains. Large cell density tended to fall then plateau in controls. In contrast, in *mdx* thymuses large cell density rose slightly then dropped. Results show that myoid cells appear capable of responding to crush injury. However, any destination outside the thymus and the myoid cell contribution to repair remain to be determined.

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LIST OF ABBREVIATIONS

AAS	aminoalkylsilane
Ab	antibody
AChR	acetylcholine receptor
AP	alkaline phosphatase
b	basic
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
ß-Gal	beta-galatosidase
BrdU	5-bromo-2'-deoxyuridine
C57	C57Bl/10ScSn mouse strain
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DMD	Duchenne muscular dystrophy
DMP-30	2,4,6-tri(dimethylaminomethyl)phenol
DTT	dithiothreitol
DDSA	dodecenyl succinic anhydride
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
ER	epithelioreticular cell
FGF	fibroblast growth factor
GF	growth factor
^3H	tritiated thymidine
HLH	helix-loop-helix
HRP	horse radish peroxidase
IGF	insulin-like growth factor
IHC	immunohistochemistry
ISH	*in situ* hybridisation
LM	light microscopy
MG	myasthenia gravis
MIR	main immunogenic region
mpc	muscle precursor cell
MRF	myogenic regulatory factor
NAA	natural autoantibodies
NMA	nadic methyl anhydride
NBT	nitroblue tetrazolium salt
PBS	phosphate buffered saline
^{32}P	radioactive phosphorus
PO	propylene oxide
RAG	autoradiography
^{35}S	radioactive sulphur
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SSC	standard saline citrate
SW	Swiss Webster mouse strain
TA	tibialis anterior
TE	Tris ethylenediaminetetraacetic acid
TEM	transmission electron miscroscopy
TGF-ß	transforming growth factor-beta
TnT	troponin T

1. INTRODUCTION

The ability to form new muscle is important for the maintenance of muscle caused by daily wear and tear, hypertrophy from active usage, repair after damage by injury and in the prevention or minimization of atrophy due to developmental or degenerative muscular diseases. In the adult, this process is termed regeneration, the subject of many investigations. Knowledge pertaining to the ability of skeletal muscle to regenerate has also come from close examination of muscle as it develops.

Satellite cells are the known primary contributors of muscle precursor cells (mpcs). The focus of this study is to determine whether myoid cells, a subpopulation of cells in vertebrate thymuses, are a secondary source of mpcs. Finding reliable means by which to identify myoid cells is fundamental for determining the distribution pattern and number of such cells in the thymus.

Myogenic regulatory factors (MRFs) are a group of genes that control muscle development. MRF expression occurs early in development in all cells with the skeletal muscle phenotype, thus making them a prime candidate to aid in the detection of myoid cells, assuming they are actually myogenic in origin. Proteins specific to striated muscle could also serve as suitable markers, but may target fewer cells depending on when the proteins are expressed during the developmental process.

The *mdx* mouse is the animal model of Duchenne muscular dystrophy (DMD). Both have an X-linked mutation that results in the absence of dystrophin, a structural protein important in maintaining stability during muscle contraction. However, whereas DMD is fatal in humans, the *mdx* mouse does not die from dystrophy. Muscles of the *mdx* mouse have an enormous capacity for repair, which continually alternates with cycles of damage, making it particularly useful in the examination of muscle regeneration.

This study attempts to assess the potential contribution of myoid cells to the process of muscle regeneration in control and *mdx* mice.

2. REVIEW OF LITERATURE

2.1 THYMUS

2.1.1 Ontogeny of the Thymus

The thymus in mammals arises from the endoderm of the third pharyngeal pouch (left and right), during the sixth week of development in humans. The pouches migrate caudally and come to rest in the mediastinum, where they partially fuse to form a single gland. Stem cells of mesenchymal origin in embryonic bone marrow migrate to populate the primordial thymus. Once there, the stem cells undergo many divisions to form vast numbers of T lymphocytes (the "T" indicates differentiation in the thymus as distinct from the antibody-producing "B" lymphocytes, in reference to the bursa of Fabricius in birds, where B cells mature). The majority of T lymphocytes (as high as 90% of cells in the thymus) undergo the process of apoptosis, or cell death, and are subsequently phagocytosed by macrophages in the thymus. It is unknown why this phenomenon occurs or how T cells are marked for it. Currently it is thought that apoptosis might function to eliminate defective lymphocytes, such as those that acquire a specificity for the self (see section 2.1.5.1) or cells not developing any functional receptors. In other words, apoptosis is potentially a safeguard to prevent the attack against self elements by the immune response (Paulsen, 1993; Benjamini and Leskowitz, 1991). The processing of T cells occurs just before birth and continues for a short time afterwards (only a few months).

The thymus responds to growth hormone, which promotes its overall development, while adrenocorticotropic hormone and corticosteroids lessen the proliferative capacity of thymic lymphocytes and thin the outer cortex (Paulsen, 1993). The thymus continues to grow, reaching a maximum stage of development in early adolescence (Moore, 1989; Benjamini and Leskowitz, 1991). The thymus also produces hormones. It is believed that epithelioreticular cells (see section 2.1.3) in the thymic medulla, which contain granulated cytoplasm, synthesize and secrete the thymic hormones. Thymic hormones include thymosin, thymic humeral factor, thymic factor and thymopoietin, the principle actions of which enhance

the proliferation and maturation of lymphocytes throughout the body.

2.1.2 Thymus Structure

The thymus is one of the primary organs of the lymphatic system. This bi-lobed structure of lymphoid tissue, located in the superior mediastinum and anterior to the great vessels of the heart, is encapsulated by a thin layer of connective tissue. Extensions of the capsule, called septa, divide each lobe into many lobules. The septa carry the vascular and nerve supplies to the tissue of this organ. Histologically, the thymus can be separated into a darker-staining peripheral cortex and a lighter-staining central region or medulla. The cortex is composed of densely-packed cells. The medulla consists of a more scattered collection of cells. Septa do not extend beyond the cortico-medullary junction and hence the lobular divisions are incomplete, leaving the medulla a single continuous core.

2.1.3 Cell Composition

The thymus is a collection of several cell types, with slight variation among species. The principle cell of lymphoid tissue is the lymphocyte. With respect to the thymus, these cells may also be referred to as thymocytes. Lymphocytes can be categorized on the basis of function, but with standard light microscopy they are virtually indistinguishable. Aggregations of cell surface proteins serve as markers to identify the various phenotypes by immunochemistry with monoclonal antibodies. The nomenclature for the surface markers are the letters "CD", the abbreviation for "cluster determinant", followed by a number, which indicates the sequence in which the marker was discovered (Benjamini and Leskowitz, 1991). Other cells that have been found in the mammalian thymus include macrophages, fibroblasts, adipocytes, plasma cells, interdigitating cells, mast cells and epithelioreticular cells (Junqueira *et al.*, 1992; Crouse *et al.*, 1985; Kaiserling *et al.*, 1974). A subgroup of the last type of cell forms Hassall's corpuscles, characteristic of the thymic medulla of humans and dogs, but not of mice or rats (Crouse *et al.*, 1985), although the exact function of the corpuscles is unknown.

Epithelioreticular (ER) cells form a supportive framework of the thymus and have an endodermal

origin while the framework of other lymphatic organs is mesodermal in origin. Reticular fibers are not secreted by the ER cells, as is the case with other lymphatic tissue. Instead, stellate ER cells, scattered throughout the organ, are connected to each other by desmosomes. As proliferation of lymphocytes takes place, the distance between epithelioreticular cells increases. However, due to the strength of desmosomes, the scaffold cells remain connected as a meshwork in which the other cellular components are suspended (Paulsen, 1993).

Since the late nineteenth century, researchers have also been aware of another population of cells in the thymus which are similar in appearance to young striated muscle cells and denoted myoid cells by Hammar (1905, in Van de Velde and Friedman, 1970). These cells are discussed below (section 2.1.4).

2.1.4 Myoid Cells

2.1.4.1 History

Myoid cells are a constituent cell type of the thymus and were first described in the late 19th century by Mayer (1888, in Puchtler, 1975) as muscle-like cells in the thymuses of frogs and salamanders. Hammar (1905) observed cells with characteristics of striated muscle in humans, but noted the resemblance was not exact and hence termed them myoid cells. Since the discovery of myoid cells, they have been found in both normal and abnormal thymuses and in numerous vertebrate species, including frogs, snakes, turtles, chickens, quail, guinea pigs, rat, mice, and humans. Reports have consistently described myoid cells as round, oval or elongated in shape, and often with visible cross-striations, appearing alone or in clusters. The majority of myoid cells are reported to be in the medulla; only a small proportion of the total number are described in the cortex.

Myoid cells have been observed at a variety of developmental stages. Mature cells have myofilaments arranged in the classical sarcomeric pattern of skeletal muscle, with discernible A-, I-, and Z-bands, and seem to be found more commonly in the lower vertebrates, such as amphibians and birds (Raviola and Raviola, 1967; Töró *et al.*, 1969). In immature cells, often in higher vertebrates, the arrangement of contractile proteins is not as advanced or regular, instead appearing scattered throughout the

cytoplasm. Humans have relatively few myoid cells in the thymus in comparison to other vertebrates; the greatest numbers have been found in adolescents or younger individuals (Henry, 1966), the earliest documented appearance being 8 weeks gestation. The age factor appears to hold true in other species as well; the younger the animal, the more developed (taking on a more striated appearance) and numerous myoid cells tend to be. However, myoid cells have often been reported in tumours and various disease conditions of the adult thymus (Van de Velde and Friedman, 1970; Drenkhahn *et al.*, 1979; Schluep *et al.*, 1987).

Investigators have noted extreme variation in myoid cell density, despite age-matched thymuses and even within different medullary regions of the same thymus (Puchtler *et al.*, 1975; Schluep *et al.*, 1987). In conjunction with their relative rarity, myoid cells have been difficult to characterize.

While EM analysis reveals the myofilamentous nature of myoid cells, tissue culture studies showed that contractile, striated muscle cells can be grown, en masse, from rat thymus cells (Itoh, 1983) and from the thymic reticulum of rats and mice (Wekerle *et al.*, 1975). Other non-thymic organs, such as spleen, liver and kidney, do not generate muscle cells (Wekerle *et al.*, 1975). This suggests that the thymus contains precursors of muscle cells that are not immediately apparent in sections by light microscopy examination (Grounds *et al.*, 1992a).

Myoid cells have been detected by immunochemical techniques, such as using sera of myasthenia gravis (MG) patients. These sera contain antibodies to skeletal muscle antigens and demonstrate a cross-reactivity in some thymus cells (Valikova *et al.*, 1982). A more common means of identifying myoid cells involves the use of antibodies to proteins specific to striated muscle, such as troponin T and troponin I (Schluep *et al.*, 1987; Meinl *et al.*, 1991); striated muscle myosin and actin (Drenkhahn *et al.*, 1978; Drenkhahn *et al.*, 1979), myoglobin, creatine kinase, enolase (Sato and Tamaoki, 1989) and many others. Immunohistochemistry has also shown myoid cells to be positive for acetylcholine receptors (AChR) (Schluep *et al.*, 1987; Meinl *et al.*, 1991). Most recently, *in situ* hybridisation has allowed myoid cells to be identified at an even earlier stage in development, by searching for the mRNA of myogenic regulatory factors (Grounds *et al.*, 1992b)(see section 2.3).

2.1.4.2 Origin and Function

Although myoid cells have been the subject of numerous studies, the origin of these cells is still controversial and their function remains a mystery.

Various authors believe myoid cells originate from epithelioreticular cells. Hammar (1905) argued that all 3 germ layers could give rise to muscle. Pappenheimer (1913, in Puchtler *et al.*, 1975) even claimed to have seen cultured epithelial cells transform into the myoid phenotype. However, the ability to spontaneously transform from one cell type to another was not well accepted, and contamination of the cultures with permissive mesenchyme was assumed as the explanation for those observations. Raviola and Raviola (1967) were also of the opinion that myoid cells had a reticular epithelial cell origin. Puchtler *et al.* (1975) referred to "myoepithelial cells" which contained myofibrils (later shown to be the smooth muscle type). Possessing characteristics of epithelial and muscle cells, myoepithelial cells were thought to serve as an intermediate between both. Found in Hassall's corpuscles, myoepithelial cells were considered to be in a state of degeneration or regression; although the exact function of Hassall's corpuscles is unknown, the possible involvement in the removal of apoptotic cell debris could explain this observation. Immunohisto-chemical studies by Drenkhahn *et al.* (1978) did not support the notion of an epithelial origin. Thymic epithelioreticular cells were found to express only smooth muscle myosin, while myoid cells reacted to striated muscle myosin only. Also, if a transition did take place between cell types, intermediates containing both smooth and striated myosins would be expected, but such cells were not found (Drenkhahn *et al.*, 1978). Sato and Tamaoki (1989) demonstrated that myoid cells were indeed distinctly different from epithelial and dendritic cells using IHC. Markers specific for these 2 cell types (antibodies to keratin and S-100 proteins, respectively) were not detected in myoid cells.

An alternative opinion is that myoid cells are derived from mesoderm, the primary germ layer that typically gives rise to muscle. Mesoderm is purportedly incorporated into the thymus during development, a plausible event since mesoderm and primitive thymus are adjacent to one another for a period during development (Henry, 1966; Töro *et al.*, 1969; Van de Velde and Friedman, 1970). Wekerle *et al.* (1975) argued against mesenchymal contamination of the thymus because myoid cells are not usually located near

the cortex but are in medullary regions, suggesting a true thymic source. Yet, if integrated early enough, a finite number of mesodermal cells could potentially migrate towards and into the medulla, which is compatible with the idea of secondary incorporation of myoid cells into the thymus. Seifert and Christ (1990) used chick-quail chimeras to try to ascertain myoid cell origin. Transplanted cranial somites did not contribute to myoid cell population in the thymus, but implanted prechordal head mesoderm did became part of thymus anlage (which contains myoid cells later in development).

A pluripotent stem cell (Wekerle *et al.*, 1975) and neural crest cells, capable of migration (Nakamura and Ayer-Le Lievre, 1986 in Seifert and Christ, 1990) are 2 other proposed sources of myoid cells, but neither idea has been pursued further.

The function of myoid cells remains elusive and literature on the subject is sparse. However, myoid cells have often been implicated in the etiology of autoimmune disorders, such as myasthenia gravis (MG) (Henry, 1966; Van de Velde and Friedman, 1970; Töró *et al.*, 1969; Schluep *et al.*, 1987). The nature of MG is based on the presence of autoantibodies to striated muscle and acetylcholine receptors. Evidence that conclusively establishes a relationship between myoid cells and the clinical course of MG is lacking. In fact, studies have revealed that density of myoid cells in control and myasthenic thymuses are not appreciably different (Sato and Tamaoki, 1989). Furthermore, helper T lymphocytes recognize class II major histocompatibility complex antigens, but such antigens are not detected in myoid cells, making autosensitization by these cells even more unlikely. Apoptosis of myoid cells, which would release muscle-like proteins capable of sensitizing thymic lymphocytes, was thought to be a possible mechanism. However, the same argument can also be made for the contribution of myoid cells to the development of tolerance in the thymus. Developing lymphocytes could be exposed to antigens of skeletal muscle by myoid cells, shown in close proximity to one another by EM analysis (Sato and Tamaoki, 1989), and result in the recognition of self muscle tissue (Valikova *et al.*, 1982).

A possible role for myoid cells in the proliferation and maturation of thymic cells has been suggested. Kamo *et al.* (1985) reported that thymic myoid cells released cytokines which promote lymphocyte expansion. Myoid cell involvement in the maintenance of the thymic microenvironment

necessary for the development and maturation of young T lymphocytes has also been proposed (Sato and Tamaoki, 1989).

While the distribution, development and features of myoid cells are reported, very sketchy (inadequate) and too few studies have explored the origin of myoid cells or the normal and pathological expressions of their function.

2.1.5 Function of the Thymus

As one of the central organs of the lymphatic system, the thymus is a chief player involved in the protection of the organism from invasion and foreign particulate matter.

2.1.5.1 Immune Function

The primary role of the thymus is to produce and distribute T lymphocytes to secondary lymphoid organs. In the thymus, T lymphocytes are programmed to recognize one particular antigenic determinant, also known as the epitope. The epitope is the portion of an antigen capable of binding specifically to a lymphocyte receptor, and the ability to distinguish between them is critical to the immune response, the process of elimination or inactivation of foreign substances. This protective function is accomplished either directly, through phagocytosis, or indirectly, through the release of destructive products causing lysis. T cells must also have the capacity to recognize and distinguish between the body's own macromolecules (self) and exogenous material (nonself) in order to prevent an immune response from being directed towards the organism itself. Thymic T lymphocytes may be further categorized into four subpopulations, according to the specific role performed during the immune response. These subsets include the helper T cells (which signal B cells to enhance production of antibodies); inflammatory T cells (which lead to the activation and migration of inflammatory cells such as monocytes and macrophages); cytotoxic killer T cells (which seek out and destroy the cell that expresses their target antigen via direct contact); and suppressor T cells (which down-regulate the actions of the previous three effector cells once the threat to the organism has passed) (Benjamini and Leskowitz, 1991).

In the thymic cortex, small lymphocytes are the most numerous, although medium and large

lymphocytes can also be found. Substantial lymphocyte proliferation occurs in this portion of the thymus. While differentiation of T cells begins in the cortex, the lymphocytes that survive apoptosis will migrate to the medulla. From the medulla, mature immunocompetent T lymphocytes (i.e. those able to recognize antigens) enter the circulation through venules or lymphatic vessels to supply the peripheral lymphoid organs (i.e. spleen, lymph nodes and tonsils) and other non-thymic areas (the mucosa-associated lymphoid tissue) which require lymphocytes. Further differentiation, maturation and proliferation, are antigen-driven and occur once T cells reach the thymus-dependent regions. The thymus itself is continually replenished by cells that migrate from the bone marrow.

2.1.5.2 Non-immune Function

While the previous section described the widely-accepted functions of the thymus, speculation as to an additional function will be outlined here. The presence of the myoid cell population in this organ has baffled researchers since its initial discovery in the nineteenth century. Although the function of this subgroup of thymic cells remains unknown, several reasons are proposed for their presence in the thymus. One possibility is that myoid cells, which have many features in common with striated skeletal muscle, serve as an example of self muscle to the developing thymus (Valikova *et al.*, 1982; see section 2.1.4.2). Alternatively, it has been proposed that myoid cells play a role in initiating autoimmune disorders, such as myasthenia gravis (see section 2.1.7). More recently, it was proposed that the myoid cells might function as a reservoir of muscle precursor cells (mpcs) that supplement satellite cells (see section 2.2.3), the primary mpc source (Mauro, 1961). This thesis project is intended to study the latter possibility more closely.

2.1.6 Involution

The onset of puberty triggers a degenerative process in the thymus, known as involution. The process is a natural one, occurring in mammals, fish and amphibians alike. Various factors aside from age have been identified to trigger involution, among them: the physiological state of the organism (like pregnancy or the lactation phase); disease or trauma (such as infection, malignancy, malnutrition or post-surgery), drugs (e.g. antibiotics) and stress (Clarke and MacLennan, 1986). It is also established that

estrogens and androgens accelerate involution, while in contrast, castration slows the process (Paulsen, 1993). The age-related modification of the organ includes a loss of size and weight, lower levels of thymic hormones in circulation (due to reduced production and secretion) and a decrease in the number of lymphocytes leaving the thymus (Schuurman *et al.*, 1991). However, involution does not destroy the thymus' capacity to function. Even the diminished adult thymus continues to be a source of new lymphocytes, needed for the body's continuing encounters with alien antigens.

Although involution is a well-recognized and accepted phenomenon, the mechanisms of the process and its significance remain elusive. Aronson (1993) proposes that involution is a form of natural selection, whereby the risk of developing autoimmune disease is diminished. In this model, the major concern is that foreign agents may present with antigens similar to those of the host. In this scenario, it is possible to speculate that the immune response, originally out to rid the organism of invading material, might turn to attack host tissue, with its similar but not identical complement of antigens. This is prevented by natural autoantibodies (NAA), which are produced by the body and thought to "mask" the potentially cross-reactive nonself antigens (Cohen and Cooke, 1986). The NAA act as opsonins, defined as markers that enhance phagocytosis of the particulate matter that express them. NAA target foreign material, thus directing T cell cytotoxicity away from the host organism's tissue. The hypothesis also incorporates the assumption that cell surface markers change with time. As the body ages, the number of modified self epitopes builds up from the ongoing exposure to DNA-damaging factors in the environment. NAA counts remain unchanged in the Buffalo rat model, which does not experience thymic involution, suggesting that the thymus is involved in the suppression of NAA production (Hirokawa *et al.*, 1990). Thus, involution would release thymic inhibition at a time when the demand for more and more NAA is high (Aronson, 1993).

An example of the ever-changing cell surface markers can be found on lymphocytes. Lymphocytes produced later in life can be distinguished from those made earlier, by the surface ratio of CD4/CD8 epitopes (the value is greater in lymphocytes produced by older animals because of fewer CD8 markers). The majority of the later T cells are of the memory type, which assist the body's immune response to second and subsequent confrontations by antigens. The corollary is that there is a decline in the numbers

of naive cells with the ability to react to modified self and new foreign antigens. This decrease in overall cytotoxicity of T cells could be considered beneficial to an aging population of cells, with higher levels of time-altered self antigens (Aronson, 1993). In essence, Aronson (1993) proposes a trade-off: to reduce the risk of developing self-reactivity, where self macromolecules become the target of immune attack, the overall effectiveness of the immune response to react to antigenic encounters of the future is diminished. The true reasons for the complex process of thymic involution are still a mystery, requiring further attention and investigation.

2.1.7 Thymus and Myasthenia Gravis

As with any part of a living system, the thymus is susceptible to abnormal states, both structural and functional. Pathological conditions of the thymus will not be reviewed here, however, the potential role of the thymus in inducing the disease myasthenia gravis has been the subject of vigorous investigation and will be discussed in this section.

Myasthenia gravis (MG) is a neuromuscular autoimmune disorder in which antibodies specific to acetylcholine receptors (AChR) are produced. These autoantibodies attack AChR in muscle throughout the body, and individuals afflicted with this condition are easily fatigued and weak. It was noted that the majority of MG patients (80-90%) also had a concomitant pathological change to the thymus (Müller-Hermelink and Marx, 1994; Melms *et al.*, 1988; Kirchner *et al.*, 1988; Schluep *et al.*, 1987). The thymus was implicated in the pathogenesis of this disease by Henry (1966) and continues to be the focus of investigation today for several reasons. Firstly, myoid cells are the only thymic cell type shown to express complete AChR (thymic epithelial cells have also been reported to have AChR on the cell surface but these lack the MIR, or main immunogenic region)(Kirchner *et al.*, 1988). It is thought that the myoid cells provide the AChR to which T lymphocytes become sensitized (Melms *et al.*, 1988; Wekerle *et al.*, 1981). Subsequent presentation of the AChR-antigen to B cells, also found in the thymus, triggers the production of AChR-specific antibodies (Vincent *et al.*, 1978 in Schönbeck *et al.*, 1992). An alternate proposal was that the myoid cells themselves were the intended target of this immune response. In other words, the

body's natural defences considered myoid cells to be ectopic muscle and responded accordingly (Wekerle *et al.*, 1978). In either case, it is the myoid cells that seem to be important for supplying AChR for interaction with immunocompetent lymphocytes. More recently, Schönbeck *et al.* (1992) transplanted thymus from human subjects with MG into SCID (severe combined immunodeficiency) mice, which lack any lymphocyte complement. Antibodies to AChR were found in the serum of these mice, indicating that the thymic graft appears to have all the essential components to produce the MG autoreaction: the antigen, antigen-presenting cells (T lymphocytes) and antibody-producing cells (B lymphocytes) (Schönbeck *et al.*, 1992). Curiously, the mice did not develop clinical symptoms of MG which the authors explained as the result of the robust resilience to manipulation that mice possess.

It should be noted that myoid cells have been demonstrated in the thymuses of the majority of the population. However, only a small fraction of people ever become myasthenic. This discrepancy argues in favour of the myoid cell presence in the thymus as being the normal condition (Van de Velde and Friedman, 1970). Furthermore, thymectomies performed early in the course of the disease have been shown to be beneficial to MG sufferers, but the return of MG symptoms in some individuals has also been reported. Together, these points suggest that other factors aside from myoid cells must be involved. To date, the thymic ties to muscle and the tempered success of thymectomies are the best leads investigators have in fighting myasthenia gravis. As such, further research into the possible involvement of the thymus in the etiology of myasthenia gravis continues. In addition, the present study examines the more basic question of myoid cell populations during development and in a non-autoimmune condition.

2.1.8 Alkaline Phosphatase

Alkaline phosphatase (AP) is an enzyme found in cells of many vertebrate species. The action of AP is to hydrolyse a phosphate group. This functional group cleavage can occur on an assortment of compounds and at least nine isoforms of the enzyme exist in humans. The distribution of AP varies among the different species. For instance, in humans the distribution is ubiquitous, being found in such tissues as bone and cartilage, the placenta and the intestine. However, the inclusion of this topic herein is due to the

presence of AP in the cells of the thymus (Meinl *et al.*, 1991). The precise function of AP in the thymus is unknown, but because the *in situ* technique performed on thymic tissue (in the present study) used an antibody conjugated to AP, it was critical to eliminate the endogenous AP activity in the thymus. This AP inhibition was attempted by treating the tissue sections prior to hybridisation with levamisole, a non-competitive inhibitor of AP. Levamisole has been found to have differential affinities to the various isoenzymes that exist and therefore the degree to which levamisole can act as an effective inhibitor is also variable (Latker *et al.*, 1987).

The thymus is a key component in the immune system and serves to protect an individual by producing cells that can combat foreign material in the body or invading organisms. Often less valued later in life after its naturally-occurring atrophic event, it should be noted that this organ continues to function well into adulthood. Although the thymus' primary function is well-known, secondary functions, such as possible involvement in thymic disorders, may yet be elucidated with further investigation.

2.2 DEVELOPMENT OF SKELETAL MUSCLE

2.2.1 Origin of Striated Muscle Cells

Skeletal muscle arises from the mesoderm, one of the three primary germ layers of the developing embryo. During embryogenesis, longitudinal columns are formed by the mesoderm on either side of the neural tube and the notochord, and are called the paraxial (somitic) mesoderm (Moore, 1989; Gilbert, 1991). The paraxial mesoderm then divides, in a rostral-caudal direction, into many cuboidal segments called somites. Each somite is populated with mesenchymal cells and consists of two regions: the dermomyotome and the sclerotome. The dermomyotome further gives rise to the dermatome and the myotome. It is the myotomal layer of the somite from which the majority of the muscles in the mammalian organism are derived, hence cells in this region have been termed myogenic progenitor cells (Olson, 1992a). Progenitor cells in the lateral regions of the somites migrate out into growing limb buds to populate the future muscles of the limbs (Stockdale, 1992).

2.2.2 Stages of Myogenesis

Stockdale *et al.* (1989) suggested that there are four instances during an organism's lifespan when muscle formation occurs. The first muscle fibers are formed in the myotome. Primary fibers follow as embryonic development progresses. Secondary fibers develop in the fetal stage, dependent upon nervous input. Finally, regeneration and repair can occur in mature muscles of the adult. The development of muscle at each of these stages is comprised of four steps: determination, proliferation, differentiation and maturation (Bishopric *et al.*, 1992).

2.2.2.1 Determination

The generation of muscle begins when mesenchymal cells of somites receive appropriate cues which direct their commitment to the muscle cell lineage. This is known as determination. *myd* is a gene which, when expressed is thought to be one of the signals involved in the determination process, since its expression causes non-muscle cells to show the myogenic phenotype (see section 2.3). The Pax-3 gene is

considered to be a likely candidate for a similar role in limb muscle development (see section 2.3.7). Other lineage determination genes have yet to be identified. Changes conferred on the cell during determination are heritable, irreversible and occur prior to the display of any observable phenotype by the cell (Stockdale *et al.*, 1989). Cells thus destined for the muscle cell fate are referred to as presumptive myoblasts. Collectively, myogenic progenitor cells and presumptive myoblasts can be termed muscle precursor cells, or mpcs. Essentially, an mpc is any mononucleated cell which can participate in the muscle program.

Mpcs express the MyoD family of transcription factors, a set of myogenic regulatory genes that are of paramount importance to myogenesis (see section 2.3). Expressed in somites long before the muscle phenotype is observed, the muscle regulatory factors are responsible for the activation of terminal differentiation genes in muscle (see section 2.2.2.3). Moreover, the members of this gene family positively autoregulate their own expression as well as each other's (Olson, 1992a), in effect sustaining the myogenic program in muscle cells during determination.

2.2.2.2 Proliferation

Mpcs undergo one or more (McGeachie *et al.*, 1993) mitotic divisions to ensure a large supply of precursors is available for growth and development. The significance of the proliferation step stems from the observation that nuclei in mature muscle fibers have withdrawn from the cell cycle and cannot divide again.

2.2.2.3 Differentiation

Through the process of differentiation, developing muscle cells undergo changes of both a biochemical and morphological nature. This phase is characterized by the expression of muscle-specific genes, such as α-cardiac actin, followed by the production of structural and contractile proteins, which lead to the cell assuming a spindle-like shape. A particular phenotype, in this case the skeletal muscle phenotype, is displayed by the cell. These cells are then termed myoblasts.

Myoblasts, or immature muscle cells, aggregate and align into parallel columns. Fusion with neighbouring myoblasts creates a syncytium, or myotube, with many central nuclei. Autoradiographic studies have shown that myotubes do not take up any tritiated thymidine, indicating that DNA synthesis is

no longer taking place in myotube nuclei. Thus, nuclei that have taken part in the fusion event are postmitotic and can no longer contribute to the precursor pool of cells.

2.2.2.4 Maturation

During the maturation phase, myotubes are transformed into mature or fully differentiated muscle fibers. Some of the more prominent changes include arrangement of contractile filaments into orderly hexagonal arrays, sarcomeric development, innervation, further protein synthesis, growth (increased length and girth of myotubes, an increased number of nuclei through additional myoblast fusion events) and migration of centrally-located nuclei to the periphery. A mature myotube, called a myofiber, is the fundamental unit of skeletal muscle.

2.2.3 Satellite Cells

No discussion on muscle development would be complete without the inclusion of satellite cells. Satellite cells, believed to be a subpopulation of the myoblast lineage, are a group of cells nestled in the tissue adjacent to the muscle fiber. By definition, satellite cells lie between the sarcolemma and the external lamina. Because of their peripheral location, satellite cells are difficult to distinguish from the peripheral nuclei of the muscle fiber using conventional light microscopy. Positive identification of satellite cells can only be achieved using electron microscopy. It is generally accepted that satellite cells are the primary contributors of mpcs to the process of muscle regeneration and growth in the adult individual; they are quiescent until required (Campion, 1984).

2.2.4 Structure of Skeletal Muscle

The long cylindrical muscle fiber is the basic cellular component of striated muscle. Each muscle fiber is surrounded by connective tissue called endomysium, which includes the external lamina synthesized by the fiber. A fasciculus is a group of muscle fibers enclosed as a bundle by a slightly thicker connective tissue layer, the perimysium. A whole muscle, a collection of fasciculi that work together and share a single main action, is bounded by the epimysium (Williams *et al.*, 1989).

Internally, each myofiber consists of many myofibrils in the sarcoplasm. Myofibrils are bundles of two major myofilaments, actin and myosin, called contractile proteins. The orderly arrangement of the myofilaments produces the characteristic banded appearance of striated muscle (Williams *et al.*, 1989). Increased synthesis of myofilament proteins and myofibrils in the central region of the myofiber displaces the myotube nuclei further towards the periphery of the cell during development.

2.2.5 Sliding Filament Model

Myofilaments are organized into repeated contractile units called sarcomeres. The distinctive light and dark bands observed in muscle correspond to the precise overlapping assembly of actin (thin) and myosin (thick) filaments, respectively. As a muscle contracts, no change in filament length is observed in EM and X-ray diffraction studies. This observation led to the proposal that thick and thin filaments slide past one another during contraction. In the presence of ATP and Ca^{2+}, and regulated b interplay with troponins and tropomyosins, the numerous cross-bridges extending from the thick filaments attach to adjacent thin filaments (actin) and pull them towards the centre of the sarcomere. This pull is the driving force behind muscular contractions.

2.2.6 Fiber Types

Most skeletal muscle fibers can be classified into one of three main categories: either type I, IIA or IIB. The distinctions are based on the isoform of myosin and many other proteins (e.g. ATPases, troponins and tropomyosin) that are expressed, the primary process of producing energy for contraction (glycolytic or oxidative), how fast a contraction can take place, and how easily a muscle tires. Fiber type is distinguished using ATPase histochemistry and more recently, anti-myosin immunohistochemistry (Williams *et al.*, 1989; Stockdale, 1990).

Type I muscle fibers are known as red, slow-twitch fibers. These are slow-twitch, oxidative fibers that can sustain repeated contraction cycles with little or no fatigue. The red colour observed is caused by the high myoglobin content in type I fibers. Type II fibers, also referred to as fast-twitch fibers are further

subdivided into two groups. The type IIA group (intermediate fibers) are fast-twitch, oxidative and glycolytic fibers; type IIB fibers are mainly anaerobic, fast glycolytic fibers. Overall, type II fibers are less resistant to fatigue than type I fibers, with type IIB fibers tiring the easiest.

Typically a mix of type I and II fibers is found in a given muscle, although one type is usually dominant (dependent on the functional usage of the muscle as a whole). In general, type I fibers are found in postural muscles, and type II fibers in muscles that produce great force during a brief timespan (Williams *et al.*, 1989).

Innervation regulates contraction rate and other muscle properties to a very large extent in the organization of motor units (a group of fibers innervated by a single motor neuron). Other factors also determine fiber type and muscle contractile properties including training, disuse, metabolism and endocrine factors. More recently, myogenic regulatory factors have also been implicated in the search for the mechanism whereby muscle fiber diversity is achieved. Hughes *et al.* (1993) showed differential expression of MyoD and myogenin, in fast and slow fibers, respectively, in the adult rat muscle, although the findings are still controversial.

2.2.7 Markers of Muscle

Since the structure of muscle precursor cells is not unique under the light microscopy particularly, various means are available that increase our ability to identify myogenic cells. To date, the most reliable marker of the earliest identifiable mpcs is the presence of transcripts from any member of the MyoD family of transcription factors. These transcripts have been found in muscle precursor cells and myotubes (Grounds *et al.*, 1992b) and mature muscle fibers (Hughes *et al.*, 1993). Preliminary evidence suggests that Pax-3, a regulatory gene of nervous system development, may be a molecular marker for the migrating population of mpcs in the myotome. During early embryogenesis, these migratory mpcs destined for the limb buds demonstrate the presence of Pax-3 mRNA prior to the expression of any myogenic regulatory factors (Bober *et al.*, 1994).

Aggregation and adhesion of myoblasts precede the process of fusion. This cell-cell interaction

is believed to be moderated by the neural cell adhesion molecule, or NCAM (Knudsen *et al.*, 1990). Muscle-specific proteins, both structural and contractile, can also be used to identify muscle cells through *in situ* hybridisation (Ontell *et al.*, 1993) and immunocytochemistry (Atsuta *et al.*, 1993). Proliferating cell nuclear antigen (PCNA) has been used to identify satellite cells at the time of their activation (Johnson and Allen, 1993). However, PCNA is not exclusive to muscle. Distinctions between different activated cell types is therefore not possible using PCNA immunostaining without dual identification because PCNA is a marker of all cells entering the S phase of the cell cycle.

It is clear that in order for myogenesis to proceed, it must take place in conjunction with the cooperation and careful orchestration of numerous factors, such as the nervous and vascular systems of the body. The process of muscle formation is a balance between the opposing forces of proliferation and differentiation. Many of the factors maintaining that balance plus the cellular and molecular cues directing the process one way or the other, have yet to be determined. The activation of a family of myogenic regulators which ultimately leads to the completion of the myogenic program (Olson, 1992b; Dias *et al.*, 1994) has provided substantial insight.

2.3 MYOGENIC FACTORS

2.3.1 The Gene Family

Myogenesis is thought to be under the control of a recently discovered family of regulatory genes. This new family, often referred to as the MyoD family of regulatory transcription factors (consisting of the four genes MyoD, myogenin, myf-5 and MRF4), has contributed significantly to the knowledge of events that control the process of muscle development (reviewed in Dias *et al.*, 1994).

The first of the myogenic factors to be cloned was MyoD, for myoblast determination gene. Davis *et al.* (1987) isolated MyoD from the mouse and found that several non-muscle cell lines could be induced to express the muscle phenotype after transfection with the MyoD cDNA.

Emerson and colleagues described another gene, *myd*, that is sometimes considered part of the MyoD family (Pinney *et al.*, 1988). *myd* is functionally similar to MyoD, in that the expression of a single *myd* locus is sufficient to activate the myogenic program in non-muscle tissue. Although the role of the gene in muscle development will not be ascertained until difficulties in the isolation and characterization of *myd* are overcome, *myd* is distinct from MyoD, as determined by Southern blot analysis of DNA.

Myogenin, a third member of the family of transcription factors, was isolated from the mouse (Wright *et al.*, 1989; Edmonson and Olson, 1989).

Other regulatory transcription factors known to date, myf-5 from humans (Braun *et al.*, 1989) and MRF4 from the rat, also known as herculin (mouse) or myf-6 (human) (Rhodes and Konieczny, 1989; Braun *et al.*, 1990; Miner and Wold, 1990) were subsequently identified.

Of the three types of muscle (smooth, cardiac and skeletal) the above related yet distinct regulatory factors are found exclusively in the cells of skeletal musculature, despite the expression of common proteins required in functional sarcomeres and contractile processes in all three. The family of muscle regulatory genes expresses nuclear phosphoproteins that have the ability to activate the muscle differentiation program in non-myogenic cell lines (e.g. C3H10T½ cells, a mouse fibroblast cell line), which then express muscle-specific genes, such as α-actin, muscle creatine kinase and troponin T, for example. Converted cells can

originate from any of the three germ layers, although some variation in the degree of myogenic conversion is apparent among the different cell types (Olson, 1993).

Recently, Grounds *et al.* (1992a) identified MyoD and myogenin transcripts in myoid cells of the thymus using *in situ* hybridisation. This was the first report of the expression of muscle regulatory factor transcripts by tissue other than skeletal muscle in a higher vertebrate.

2.3.2 Shared Properties

The proteins translated from myogenic regulatory genes have approximately 80% sequence homology in a segment of roughly 70 amino acids. Within that segment, two regions of are particular interest: the basic and helix-loop-helix domains. The basic (b) domain, so named because of the many basic amino acid residues it contains, is involved in DNA-specific binding. Situated adjacent to the basic region is the helix-loop-helix (HLH) region. The HLH region is composed of two amphipathic alpha-helices (consisting of 12-15 residues each) and separated by a non-helical loop of variable length (Olson, 1990; Benezra *et al.*, 1990). The HLH region mediates dimerization of the protein products of the transcription factors. With the exception of a few short sequences, there is little similarity between the muscle regulatory proteins outside the bHLH region of homology.

2.3.2.1 Conservation in Species

All four members of the MyoD family have been found in several vertebrate systems, including humans, mice and rats, frogs (*Xenopus*) and birds (quail and chicken). In invertebrates, it seems that a single master gene can account for muscle development. The invertebrate gene shares greatest sequence homology (about 70%) with MyoD. MyoD homologues have also been identified in *Caenorhabditis elegans*, *Drosophila* and sea urchins (Olson, 1993). It should be noted that the MyoD-like gene may not be the only factor involved in initiation and maintenance of myogenesis in invertebrates. Gene knockout experiments that targeted hlh-1, the MyoD homologue in *C. elegans*, demonstrated that muscle formation still occurred, suggesting that another gene or genes must be compensating for the loss. The lone myogenic gene in *Drosophila* is only detectable during muscle development. After differentiation, the MyoD-like gene

is no longer expressed. It was concluded that some other gene was responsible for the muscle program after that time point (Olson, 1993). Nonetheless, the bHLH region is a highly conserved motif of muscle-specific factors in both vertebrates and invertebrates (Olson, 1990).

2.3.3 Dimerization and DNA Binding

The MyoD family is thought to be responsible for the initiation of the muscle program in cells. The HLH motif of these genes acts as an interface for dimerization of HLH proteins. Dimerization can occur between two identical proteins (homodimerization) or among different proteins (heterodimerization). The efficiency of homodimer formation is quite low. Consequently, homodimers are not thought to be major players in the myogenic event (Olson, 1990). Further evidence to support this idea, is that heterodimerization with certain partners increases the affinity of HLH protein complexes for DNA binding. The usual partners of muscle transcription factor proteins in dimers belong to a family known collectively as E proteins, which are immunoglobulin enhancers. Myogenin and MyoD dimerize with ubiquitously expressed E12 and E47 proteins, products of alternative splicing of the E2A gene (Murre *et al.*, 1991 in Biben, 1993). The HLH domain is especially important because HLH proteins cannot proceed to bind to DNA unless they are in the dimerized form.

The basic region recognizes the DNA consensus sequence, CANNTG (where N can be any nucleotide), also called an E box (Church *et al.*, 1985 in Olson, 1990). A protein complex can bind to an E box either directly or indirectly, through an intermediate transcription factor, such as muscle-specific enhancer-binding factor 2, or MEF-2 (Olson, 1992b). Once bound to an E box, protein complexes can then activate the promoter of a muscle-specific gene, enhancing the transcription of that gene. The control regions of genes specific to skeletal muscle contain E boxes in most cases. In experiments where the E box (or E boxes) is inactivated, transcription of muscle-specific genes is also eliminated. However, E boxes by themselves are not enough to generate transcription, since the cooperation of other cellular factors is also required (Olson, 1993).

2.3.4 Pattern of Expression

MyoD, myogenin, myf-5 and MRF4 demonstrate a specific temporal and spatial expression pattern *in vivo* (Sassoon *et al.*, 1989; Hannon *et al.*, 1992; reviewed in Biben, 1993). Using the method of reverse transcriptase-polymerase chain reaction in a study of the developing mouse embryo, Hannon *et al.* (1992) determined that myf-5 is the first muscle regulatory gene to be expressed at 7.5 days post coitum (dpc) in the dermomyotome of the developing embryo. myf-5 expression precedes the formation of the myotome, which occurs at 8.0 dpc, as well as the production of any muscle-specific transcripts (Hannon *et al.*, 1992). The myogenin gene product appears in somites at 8.5 dpc (Hannon *et al.*, 1992), coinciding with the earliest detection of structural genes, such as α-cardiac actin expression (Biben, 1993). MyoD transcripts are detected two days later at 10.5 dpc. MRF4 protein displays a bi-phasic expression pattern. In the whole embryo, the MRF4 gene product is observed transiently on day 10.5 of embryonic development (Hannon *et al.*, 1992), then levels drop until embryonic day 15.5, at which time MRF4 transcript levels rise a second time (Biben, 1993). A decline in Myf-5 transcripts is also noted at the same time as MRF4 disappears, and myf-5 expression becomes undetectable by day 14.0 of gestation (Buckingham *et al.*, 1992).

In vivo studies have also illustrated that expression of MyoD, myogenin and myf-5 is high in fetal skeletal muscle (Wright *et al.*, 1989; Braun *et al.*, 1989; Davis *et al.*, 1987; Rhodes and Konieczny, 1989). The mRNA levels of these myogenic factors fall drastically after the first week of development in the rat but their presence is still detectable in adult musculature (Hughes *et al.*, 1993). MRF4 activation is observed transiently in the developing embryo, but in mature skeletal muscles, the amount of MRF4 mRNA transcripts exceeds those of the other three myogenic transcription factors (Rhodes and Konieczny, 1989; Miner and Wold, 1990).

In developing limb musculature, myf-5 is again the first transcription factor to be expressed, appearing in the forelimb bud at 9.5 dpc and the hindlimb bud at 11.5 dpc (Hannon *et al.*,1992). Myogenin, MyoD and MRF4 are all expressed simultaneously at 10.5 dpc in the forelimb bud (Hannon *et al.*, 1992). In the hindlimb bud, expression of myogenin and MRF4 can be detected at 11.5 dpc, while MyoD expression is not apparent until 12.5 dpc (Hannon *et al.*, 1992).

In vitro development studies have also contributed to our knowledge of the temporal and spatial expression pattern demonstrated by the muscle transcription factors. Smith *et al.* (1993) examined the mRNA and protein levels of the MyoD family at various times points in four different stages of mouse development (somitic, embryonic, fetal and newborn). Immunolocalization studies of cultured myoblasts determined that a similar pattern of expression of the myogenic regulatory factors was found in embryonic, fetal and neonatal cells: MyoD transcripts were the first to be transcribed, followed by myogenin, myf-5 and finally MRF4. A similar pattern was observed for the protein products of these genes, and was confirmed by RT-PCR and Northern blot analysis by the same investigators. Somitic cell cultures gave markedly different results. The expression of myf-5 was detected first in such cultures, with subsequent activation of MyoD and myogenin. Although MRF4 was observed in somitic cell cultures, in contrast to the cultured myoblasts, the above *in vitro* findings are consistent with the *in vivo* studies described earlier.

More recently, Smith *et al.* (1994) investigated the temporal pattern of rat skeletal muscle satellite cells with RT-PCR. The determined order of mRNA expression was MyoD, myf-5 and MRF4, and finally, myogenin, with myf-5 levels decreasing by the time myotube formation takes place.

Collectively, these studies show that the myogenic regulatory factors exhibit a spatiotemporal expression pattern and that distinct populations of myoblasts destined for specific fates may express these factors in different sequences. This idea is one possible explanation for the disparities encountered between *in vivo* and *in vitro* studies. Thus while *in vitro* cultures substantially enhance our understanding of the molecular basis of myogenesis, they cannot supersede the importance of *in vivo* studies because exact conditions of the muscle cells and tissues in the developing embryo cannot be replicated.

2.3.5 Regulation of the Regulators

Myogenic regulatory factors are themselves under regulation. Some of the better known mechanisms involve oncogenes, other families of factors (such as the Id family; transforming growth factor; fibroblast growth factors and insulin-like growth factors), electrical stimulation, 5-bromo-2'-deoxyuridine, as well as autoregulation. These regulators exert their effects at multiple levels of the myogenic program

(e.g. during transcription or translation of the muscle regulatory genes).

2.3.5.1 *myc* Family and other Oncogenes

Basic and HLH domains are not unique to the muscle-specific transcription factors. bHLH regions can be found in other regulatory proteins, including the *myc* family of oncogenes, in addition to other proteins (for example, see Id, section 2.3.5.2) involved in regulating cell type-specific transcription in a wide range of organisms (Murre *et al.*, 1989). Oncogenes, such as *myc*, *fos*, *jun* and *ras*, have been shown to promote cell proliferation. *ras* inhibits the myogenic program by blocking the activation of the muscle regulatory genes, although forced expression of MyoD can rescue these cells and restore the myogenic capability (reviewed in Olson, *et al.*, 1991). The mechanism of this inhibition has not been determined.

2.3.5.2 Id

Another group of proteins that contain the HLH region is the Id family. Benezra *et al.* (1990) was the first to characterize Id, an abbreviation for "inhibitor of DNA binding", and found Id was able to attenuate myogenesis. A distinguishing feature of the Id protein family is the absence of the basic region. The HLH region allows for heterodimer formation with other HLH proteins, namely the regulatory transcription factors and E proteins. Negative regulation of the muscle regulatory program is achieved because the resulting complexes are non-functional since DNA binding is not possible without the basic region. High levels of Id are expressed in proliferating myoblasts (suggesting possible involvement in embryonic growth). Down-regulation of Id occurs during differentiation (Olson, 1993), consistent with the theory that Id proteins regulate myogenesis through competition for E2A proteins, the heterodimerization partners of muscle regulatory proteins. Id's down-regulation makes the essential E proteins available for muscle transcription factors which can then form the functional heterodimers necessary for the myogenic pathway to proceed (Olson, 1990). In addition, the expression of Id and myogenic regulatory factors are mutually exclusive in cell culture studies, further evidence in support of Id's negative regulatory role in muscle formation (Wang *et al.*, 1992).

The ubiquitous distribution of Id suggests that the regulatory role of Id may not be restricted to muscle. The inhibitory effects of Id might apply to regulatory factors of other cell lineages as well. The

diminished levels of Id at the time of differentiation also suggest that Id may be important in withdrawal from the cell cycle (Benezra *et al.*, 1990).

2.3.5.3 Growth Factors (TGF-ß and FGFs)

Growth factors (GFs) are involved in cell proliferation and growth. The function of GFs is generally antagonistic to that of the MyoD family, which initiates the muscle differentiation program in cells (Olson *et al.*, 1991). GFs interfere with the differentiation of determined myoblasts by targeting myogenic regulatory gene expression. The repression of muscle-specific transcription factor activation is relieved once GF levels in the cells fall below a certain threshold.

The ubiquitously expressed transforming growth factor-ß, or TGF-ß, is the most potent myogenic inhibitor identified thus far. Originally identified as an inducer of phenotypic transformations in fibroblasts, experiments conducted in the mid-1980s in immortalized cell lines demonstrated that TGF-ß interrupts all measurable characteristics of muscle differentiation, such as fusion, elevation of creatine kinase, appearance of acetylcholine receptors and expression of muscle-specific mRNAs (Florini *et al.*, 1991). TGF-ß's mechanism of action is thought to be by the upregulation of *jun* and *ras* oncogene expression, which in turn blocks muscle development by reducing the amount of myogenin mRNA, a fundamental requirement for myoblast differentiation.

Fibroblast growth factors, or FGFs, come in two forms: acidic and basic. The basic type is more abundant and the more potent of the two FGFs in inhibiting the differentiation of muscle cells. FGFs have a mitogenic effect on tissues of mesodermal and neuroectodermal origin (Gospodarowicz, 1987). The mechanism through which FGF acts to inhibit differentiation is unknown, although the oncogenes *c-fos* and *c-myc* may be involved. Speculation also revolves around the direct suppression of myogenic regulatory genes themselves by FGFs (Vaidya *et al.*, 1989 in Florini *et al.*, 1991), although recent work in our lab (Garrett and Anderson, 1995) showed that mRNA transcripts of bFGF (basic FGF) can be co-expressed with myogenin transcripts by muscle precursor cells and myotubes *in vivo* in regenerating muscle.

MyoD and myogenin proteins are known to be phosphorylated. In addition to up-regulating oncogenes, GFs might also affect the degree of phosphorylation of the myogenic factors, which could in

turn interfere with activities requiring phosphorylated sites (Olson *et al.*, 1991).

2.3.5.4 Growth Factors (IGFs)

Insulin-like growth factors (IGFs) form a third GF family that stimulates cell proliferation. The family is comprised of 3 members: IGF-I, IGF-II and insulin. Unlike TGF-ß and FGFs, which inhibit myogenic differentiation while promoting proliferation, IGFs stimulate differentiation in muscle cells, with IGF-I being the most potent stimulator. The general characteristics of IGFs apply to both *in vivo* and *in vitro* models (reviewed in Florini *et al.*, 1991). Tissue culture experiments revealed that muscle cells were a significant producer of IGFs (although the primary source of serum IGF-I is thought to be the liver) (Florini *et al.*, 1991). However, although both liver and muscle make IGF-I in response to pituitary growth hormone (GH), the stimulatory effect of GH is more than twice as potent in muscle compared with liver (Murphy *et al.*, 1987).

Florini and colleagues (1991) suggest that IGFs act by inducing the activation of myogenin, the muscle regulatory gene most intimately associated with muscle differentiation. These investigators also suspect that IGF receptors and IGF binding proteins also have a part in mediating the stimulation of myogenic differentiation, although their exact role remains to be elucidated. And finally, IGFs have also been implicated in the elevated levels of oncogene expression, which themselves could be important in the differentiation event in muscle (see section 2.3.5.1) (Florini *et al.*, 1991).

2.3.5.5 Other Known Regulators

Eftimie *et al.* (1991) reported that mRNA levels of MyoD and myogenin declined in response to innervation. Denervation of muscle in young mice resulted in augmented quantities of MyoD and myogenin transcripts being detected. With stimulation by an external source, activation of muscle regulatory genes was attenuated. In light of these results, the authors concluded that MyoD and myogenin could be regulated by electrical activity. There is also recent evidence that the extent of muscle regulatory gene expression is fiber type specific in adult muscle (Hughes *et al.*, 1993), perhaps a reflection of contractility- or activity-dependent expression of myogenin and MyoD, or an indication of post-differentiation roles for the muscle regulatory genes in mature muscle. Future studies may provide insight as to why muscle regulatory genes

should be differentially expressed in fast and slow muscle.

Finally, 5-bromo-2'-deoxyuridine, or BrdU, has also been implicated in the experimental inhibition of myogenesis. It is thought that BrdU acts by interfering with phosphorylation of the regulatory factors, reducing the success of participation in later steps in the muscle program (Tapscott *et al.*, 1989).

2.3.6 Autoregulation

An intriguing property of the MyoD family of muscle regulatory genes is the characteristic ability to self-regulate expression (reviewed in Olson, 1991 and in Tapscott and Weintraub, 1991). Activation of any one of the muscle regulatory genes causes positive feedback which increases or continues the expression of the same gene, causing up-regulation. In addition to turning on the endogenous copy of itself, expression of a single myogenic regulator can, to some extent, activate the other members of the group (Weintraub *et al.*, 1991). This sophisticated interplay of auto- and cross-activation combined with the spatiotemporal regulation among the transcription factors serves a potentially dual function. The first possible action is to amplify transcription of the muscle regulatory genes. The second putative function is to reinforce commitment to the muscle program, making reversal unlikely once the program for commitment is started.

2.3.7 Functional Redundancy

Quantitative differences among each of the muscle regulatory factors, with respect to factors such as amino acid sequences and level of expression, have been demonstrated. Qualitatively, each of the members can induce the myogenic phenotype in non-muscle cell lines on its own (Dias *et al.*, 1994). Together, these two points suggest a functional redundancy. In recent years, genetic manipulations have produced transgenic mice with null mutations of MyoD, myf-5 and myogenin (Rudnicki *et al.*, 1992; Braun *et al.*, 1992; Nabeshima *et al.*, 1993; Hasty *et al.*, 1993). Surprisingly, mutants with the null mutation of either MyoD or myf-5 show little deficiency in muscle development. However, while MyoD mutants are viable and can produce offspring, muscle cells in the MyoD knockouts do show elevated levels of myf-5 gene transcripts. Rudnicki *et al.* (1992) concluded that MyoD normally suppresses myf-5 expression and

that in MyoD's absence, myf-5 could be up-regulated to compensate for the loss in regulatory gene expression. The myf-5 genetic knockouts show a delay (about 2 days) in myotome formation during development although myoblast differentiation did eventually occur. Myf-5 knockout mutants are not viable due to abnormal formation of the ribs (sclerotome derivative of the somitic mesoderm) which permits respiration, although no skeletal muscle abnormalities were reported (Braun *et al.*, 1992). These studies show that either MyoD or myf-5 is expendable from the muscle program without compromising normal development of muscle.

In contrast, transgenic mutant mice homozygous for a non-functional myogenin gene showed severe deficiencies in skeletal musculature, and deformities to the rib cage were also observed. However, some poorly formed fibers were observed, possibly due to the presence of a subset of myoblasts which do not require myogenin for differentiation (Nabeshima *et al.*, 1993; Hasty *et al.*, 1993). Myogenin-negative mutant mice were born alive but died shortly after. The two gene knockout studies concluded that myogenin is critical to normal muscle development *in vivo*, specifically during muscle differentiation. In contrast to MyoD or myf-5 knockout experiments, other members of the MyoD family cannot offset or substitute for the absence of myogenin. MRF4 gene knockout experiments are currently underway and preliminary results are similar to those obtained with the myf-5 mutants. The report indicates that skeletal muscle is present, but the MRF4 knockout mice do not survive, again because of abnormal rib cage formation (Buckingham, 1994).

While MyoD or myf-5 mutations alone do not disrupt the normal course of muscle development, Rudnicki *et al.* (1993) crossed the two mutant strains to generate animals with the double mutation, and further investigate the role of MyoD and myf-5. The double knockout mice, which did not survive, completely lacked skeletal muscle, demonstrating that the presence and expression of at least one of MyoD or myf-5 is essential for normal skeletal muscle formation.

The precise concentrations and interactions of the myogenic regulatory factors required for normal formation of muscle have yet to be ascertained. The importance of the MyoD family to myogenesis cannot be disputed, but it should also be emphasized that the myogenic factors are not the only elements involved

in the induction of muscle development. For instance, MyoD is said to be responsible for activating expression of such muscle-specific genes as α-actin and muscle creatine kinase. Yet transcription of these latter two genes occurs as early as embryonic day 8.5 (E8.5), before the detection of MyoD mRNA at E10.5, suggesting that something even earlier is controlling the expression of the muscle-specific protein genes. Alternatively, MyoD mRNA may be in small but very potent concentrations detectable only by RT-PCR (Lin-Jones *et al.*, 1994).

The presence of myf-5 during the early events of myogenesis lead to speculation that myf-5 might also have a role in the determination of muscle cells. Tajbakhsh and Buckingham (1994), in a tissue culture study, have shown that limb buds of mice are initially myf-5 negative (myf-5 expression occurs only after a few days in culture). This indicates that a gene upstream of myf-5, the earliest myogenic transcription factor to be expressed, has already determined this somitic subpopulation to become muscle, prior to myf-5 activation. Pax-3, a transcription factor normally associated with the formation of the nervous system, might be the gene in question.

In two recent studies, a mutation in the Pax-3 gene was reported to result in severely deformed limb muscles (Franz *et al.*, 1993; Bober *et al.*, 1994). Pax-3 expression also identified a subpopulation of cells that migrate into limb buds, making Pax-3 an even earlier marker of muscle precursor cells than the MyoD family, which is not yet expressed in the migratory cells (Bober *et al.*, 1994). Although the exact role of Pax-3 in the myogenic pathway requires further investigation, these studies illustrate the importance of other factors besides myogenic regulatory genes in early myogenesis.

Myogenic regulatory factors are critical components in the development of striated muscle. Expanding the knowledge base regarding these factors has assisted immensely in our understanding of muscle development and regeneration, and in future could conceivably lead to therapies for muscle disorders that exploit their potential.

2.4 *IN SITU* HYBRIDISATION

In situ hybridisation (ISH) is a powerful tool in the investigative arena. Conceived roughly two decades ago, this technique provides a means for the detection of nucleic acid sequences at the level of the cell, tissue or animal in its entirety. As well, spatial and temporal expression patterns of genes in an organism can be determined, an important step towards the comprehension of molecular control mechanisms of processes *in vivo*.

Although conditions should be optimized with respect to the particular nucleic acids of interest and the specific tissue in which they are found, there exists, in general, a consensus on the basic procedure of *in situ* hybridisation. Wilkinson (1992) and Sambrook *et al.* (1989) proved to be excellent sources of references for the protocols outlined in the subsequent sections.

2.4.1 General Principle

The annealing, or pairing, of homologous sequences of nucleic acids is the cornerstone of *in situ* hybridisation. A labelled sequence of nucleotides (probe) anneals, or hybridises, to a complementary sequence in the tissue (target). Specific labelling of the probe allows for subsequent visualization of the hybrid probe/target nucleotide sequences. The components and steps involved in ISH are discussed in the following sections.

2.4.2 The Probe

A probe consists of a series of nucleotides, the order of which is complementary to the sequence of interest which is located in the specimen. Probes can be either RNA or DNA, and if the latter, single- or double-stranded (Wilkinson, 1992). The discussion which follows deals with riboprobes.

RNA probes are all single-stranded. Purified RNA polymerases (e.g. T3 or T7 RNA polymerase) are used to synthesize the probe sequences from substrate nucleotides (UTP, GTP, ATP, CTP), one of which is labelled for subsequent detection. Downstream of the polymerase initiation site, the sequence of interest is subject to transcription by the enzymes. For convenience, probe sequences are usually cloned into a

plasmid vector, such that two different polymerase initiation sites flank the sequence. This allows for the production of two probes. A sense strand is made and is used as a control or negative probe, since its sequence is identical to the target sequence and annealing does not take place. An antisense probe is made in the reverse direction of transcription such that its sequence is complementary to the target sequence which permits duplex, or hybrid, formation (for further details regarding probe synthesis, see Sambrook *et al.*, 1989).

2.4.3 Labels

Probes for *in situ* hybridisation must somehow be marked so that future detection is possible. The two major types of labels in use are radioactive substances and non-radioactive molecules, both conjugated to a nucleotide (often UTP). The decision to use one over the other is dependent on personal preference, as both offer comparable degrees of sensitivity. However, growing concerns over health and waste disposal associated with radioactive systems of detection, coupled with the improvements made to non-radioactive strategies (for instance, superior resolution, see section 2.4.3.2 below) have prompted a growing movement towards the latter approach.

2.4.3.1 Radioactive Markers

The three radioisotopes in regular use are tritiated thymidine (^{3}H), and radioactive sulphur and phosphorus (^{35}S and ^{32}P, respectively). The three differ in their signal resolution, speed of development and stability of the probes (^{3}H is the most stable with subcellular resolution, but has a slow development time; the other two provide faster results, but slightly poorer resolution)(Wilkinson, 1992).

2.4.3.2 Non-radioactive Markers

Non-radioactive reporter molecules are introduced into a probe directly. These moieties have the advantage of being safer, with fast results and can be resolved at the level of a single cell. A consideration regarding the use of these molecules would be to ensure that nothing endogenous to the tissue under examination has a resemblance to the moiety, otherwise non-specific signal will be produced. This concern can be addressed as one of the tissue pretreatments prior to the actual hybridisation event during the ISH

procedure.

2.4.4 Tissues and Pretreatments

ISH can be performed on cultured cells, cell suspensions, tissue sections (frozens, wax or plastic embedded) or whole mount preparations (Wilkinson, 1992). Tissues require a fixation step in order to preserve morphology and slides must go through a subbing or silanating preparation to improve adhesion of sections to the glass during processing.

Of crucial importance to the success of ISH is the accessibility of the target sequence to the probe. A protein digest (proteinase K is most commonly used) serves to remove protein cross-linkages surrounding the target sequence which may be created during tissue fixation. The digestion thus allows for greater probe penetration. An acetic anhydride step, which reduces non-specific binding of the probe to positively-charged amino groups in the tissue, can also be incorporated. Removal of some endogenous enzyme is often critical for the ensuing detection step (see section 2.4.6.2). Tissues are put through these pretreatment steps to maximize hybridisation efficiency and decrease the production of non-specific (i.e. inappropriate positive) signal (Wilkinson, 1992). Care must also be taken to guard against loss of the target sequence due to nuclease degradation through handling, fixation and other procedures, since RNase and DNase enzymes are commonly found on ungloved hands.

2.4.5 Hybridisation and Washing

Optimal conditions are required for the hybridisation step in order to maximize the annealing of probe and target sequences. Controllable parameters include the temperature at which hybridisation takes place, the concentration of the probe and hybridisation time (Wilkinson, 1992). Higher temperatures will leave only the most complementary sequences intact (called higher stringency conditions, see below). That is, the other related but distinct sequences would be denatured. However, caution is required since higher temperatures will also approach the melting point of probe/target hybrids, thereby compromising the stability of duplexes and decreasing signal detection. There is also a point of saturation at which target sequences

are sufficiently located by the probe. Use of an amount of probe surpassing the saturation value will increase the likelihood of high levels of non-specific binding without any corresponding amplification of the intended signal (Wilkinson, 1992). Optimum hybridisation time and the best value for each of the above variables, vary with the specific tissue being examined and the precise series and sequence of pretreatments performed prior to hybridisation. Consequently, these parameters should be worked out empirically for the particular system under study.

Post-hybridisation washes are intended to eliminate any mismatched or unhybridised (single-stranded) probe that remains. Washing promotes the dissociation of probe bound to non-homologous sequences which contribute to background signal. When working with RNA probes, treatment with an RNase following hybridisation will selectively degrade any single-stranded probe (RNA probes hybridised to complimentary sequences are resistant to RNase digestion)(Wilkinson, 1992).

The stringency of a solution can also moderate the amount of non-specific binding of the probe. Stringency is based on the temperature and the concentration of monovalent cations in both hybridisation and washing solutions: the more positively charged the solution, the greater the hybrid stability (temperature was already discussed above). Thus, the lower the salt concentration and the higher the temperature, the greater the stringency. The principles of stringency may be applied during the hybridisation step or the post-hybridisation washes to attenuate the levels of non-specific binding, since these levels are the limiting factor in determining how effective (specific and high resolution) an ISH experiment has been.

2.4.6 Visualization of the Signal

Once hybridisation and the post-hybridisation washes are achieved, the probe must be detected *in situ* in the tissue or cells. The method of this visualization is dependent on the marker that labelled the probe as synthesized from nucleotides.

2.4.6.1 Autoradiography

The detection of radioactive probes is accomplished through autoradiography (Wilkinson, 1992). Briefly, the process involves covering tissue specimens (sections) on slides with liquid emulsion by dipping

(allowing for drying), exposure of coated slides at 4°C for an appropriate period of time, and finally, photographic development of the exposed silver grains.

2.4.6.2 Immunocytochemistry

Non-radioactively labelled probes are detected by high affinity immunocytochemistry. Antibodies (Ab) or binding proteins specific to the moieties are coupled or conjugated to an enzyme, fluorochrome or colloidal gold. Binding sites on proteins, which are not specific to the target moiety, are made unavailable to conjugated antibodies by competitive blocking, with solutions of small proteins (albumin, serum), before and during the application of antibodies.

The most frequently used enzymes for conjugation to antibodies are alkaline phosphatase, ß-galactosidase and peroxidase. It is imperative that the tissue of interest does not naturally possess this enzyme (otherwise the outcome is a false positive signal). Inactivation of the endogenous enzyme by treatment of tissues with excess substrate or inactivators prior to application of enzyme-conjugated antibodies typically address the problem. When the tissue is incubated with the enzyme's substrate, the reaction produces an insoluble, colour precipitate at the site of hybridisation. Depending on the stability of the enzyme used, reaction times can be extended to obtain a more substantial signal (Wilkinson, 1992).

A variation on the enzyme-substrate-colour precipitate combination is to use a chemiluminescent substrate. When such a substrate is cleaved by its enzyme, an unstable intermediate is produced. This intermediate emits light, directly proportional to the amount of enzyme-coupled Ab present, which can then be recorded on x-ray film, as in Northern blot detection of mRNA probes.

Fluorescence (of probe or Ab) can also be used to detect a signal. The advantage of fluorescence is the availability of a variety of colours, which allows for double-labelling or co-localization of two or more sequences or moieties (e.g. a protein plus an RNA sequence) within the same cell.

Another alternative is Abs conjugated to gold particles (good when high resolution is required, such as in EM work)(Wilkinson, 1992).

Digoxigenin (a steroid found exclusively in digitalis plants) and its corresponding antibody (conjugated to alkaline phosphatase, for instance) are recent developments of the moiety-Ab system.

Streptavidin-biotin complexes that form from biotinylated probes are an example of the hapten-binding protein strategy.

Following both autoradiography or immunocytochemistry, tissues are usually counter-stained and mounted under coverslips. Observations and photography can then be carried out by light microscopy (Wilkinson, 1992).

2.4.7 Experimental Controls

Although ISH is the authoritative technique for the detection and location of nucleic acid sequences, precautions to ensure specificity and sensitivity of the technique should not be neglected. *In situ* hybridisation can give misleading results, as a consequence of unanticipated homologies and experimental artifacts. Inclusion of appropriate controls, such as nuclease pretreatments or a no probe negative control in the procedure particularly during its development in a new lab or for new tissue or detection systems, will show the effectiveness of the ISH and provide confidence in any conclusions derived from the procedure.

2.5 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is a technique which can identify cell and tissue constituents and their distribution with a high degree of specificity. Originating in the early 1940s, the method removed much of the uncertainty in the field of histopathology, which at the time was largely based on subjective interpretation of results, often based on intuition and deduction.

Ideally, optimum conditions should be worked out empirically for each antibody and tissue being studied. For the basic steps and general background of immunohistochemistry, *Light Microscopy in Biology: A practical approach* (Lacey, 1989) and *An Introduction to Immunocytochemistry: current techniques and problems* (Polak and Noorden, 1987) are highly recommended reference manuals, and were referred to often for detailing the procedure in the following sections.

2.5.1 General Principle

Immunohistochemistry involves the use of labelled antibodies as specific reagents to localize antigens (cell products) within various tissue sections. The natural immunological function of an antibody is to locate the particular antigen to which it is perfectly matched and bind to it. In this manner, antigens (any foreign agent) are marked, facilitating the migration of other components of the immune response to the area to rid the organism of the potentially harmful material.

2.5.2 The Antigen

An antigen can be any molecule or substance that can react with specific components of the immune response, antibodies in particular. Antigens can have one or many reactive sites. Thus, although antibodies are produced exclusively to a specific epitope, multiple antibodies can be bound to a single antigen.

2.5.3 The Antibody

Antibodies, also known as immunoglobulins, are proteins secreted by plasma cells, which are

differentiated B lymphocytes. About 20% of the proteins circulating in human blood plasma are antibodies, capable of existing in millions of forms. When an antigen stimulates the immune response, plasma cells are triggered to form antibodies specifically reactive to that antigen. An organism can produce and contain thousands of antibodies at any given time, since it is continually being exposed in its natural environment to substances with the potential to cause an immune reaction.

Structurally, antibodies are dimers, consisting of two identical heavy chains and two identical light chains of polypeptides. The chains are held together by disulfide bonds. The binding site of the antibody is specified by the amino acid sequence of the variable region on one heavy and one light chain. The variable region is attached to the constant region of each chain, and the latter is responsible for antibody characteristics such as anchoring the protein to the cell membrane (Gilbert, 1991). In some instances, antibodies are digested by enzymes to give fragments still capable of reacting with an antigen, known as Fab fragments (antigen-binding fragment). Fab fragments are used in place of the whole antibody as the smaller size allows for amplification of labelled and active antibody without cumbersome steric hindrance at the site of binding to the antigen.

Two types of antibodies are obtained through immunization: polyclonal and monoclonal. Polyclonal antibodies are pooled antibodies produced by several different clones of stimulated plasma cells, each specifying a particular epitope on the original antigen. Monoclonal antibodies are produced by clones derived from a single plasma cell, often made from a hybridoma (cell line formed from the fusion of a normal B lymphocyte and a lymphocyte tumour cell). Specificity is extremely high in the monoclonal antibodies because their single B cell source only specifies one epitope on a particular antigen.

2.5.4 Labels

There are 3 types of labels used to visualize antibody-antigen complexes. Visualizing agents can be fluorescent, enzymatic or gold with silver enhancement. Labels are attached to at least one set of antibodies used during the IHC technique.

Fluorescent labels give fast results, requiring only incubation with a labelled antibody, mounting

and examination of results. The most common fluorescent labels are fluorescein and rhodamine. Microscopes equipped with suitable filters are requisite to see this type of label, and even then, the morphology of the surrounding non-fluorescent tissue will not be visible, unless by phase contrast.

Enzymatic labels are visualized with a reaction that produces an insoluble colour precipitate. Enzymes suitable to function in this capacity should not be those that are also endogenous to the tissue of interest, and are chosen to possess a high reaction turnover rate to produce large quantities of precipitate. Furthermore, the precipitate product must be insoluble, important for mounting purposes. Horse radish peroxidase (HRP), alkaline phosphatase (AP) and beta-galatosidase (ß-Gal) are examples of some frequently used enzymes.

Colloidal gold labelling uses antibodies attached to gold particles. Gold being a heavy metal, was first used in electron microscopy because of its electron density (Lacey, 1989). With the use of different sized gold particles conjugated to different antibodies, co-localization studies of 2 antigens or epitopes could be performed in the same section. A silver enhancement step is required to see the gold particles using light microscopy.

The choice of label to be used is a question of what is most appropriate for the situation. For example, AP should not be used in a study of the gut because high endogenous levels of AP reside in this tissue. If high sensitivity is required, the use of enzymes and colloidal gold are the more sensitive of the visualizing agents just described. Ultimately, after the above considerations are made, deciding on a label may be left to personal preference, to the species of animal in which the antibodies were raised, and to the source species of pre-conjugated antibodies labelled by the various commercial products available.

2.5.5 Application

Antigen detection can be done either directly or indirectly. The direct method has the label attached right to the antibody. The method is fast, with results available after one incubation. The more favoured method though uses an unlabelled primary antibody which is then detected by a labelled secondary antibody. The secondary antibody has specificity for the species from which the primary antibody was

obtained. The main advantage over the direct method is increased sensitivity, a consequence of multiple secondary antibodies being able to react with each first antibody, thereby amplifying the original single primary antibody detection of an epitope. The biotin-streptavidin complex is a commonly used indirect system. Biotinylated antibodies exploit the high affinity that streptavidin, a protein isolated from *Streptomyces avidinii*, has for biotin, a protein originating in the liver.

2.5.6 Controls

Immunohistochemistry procedures require both a positive and a negative control. For every antibody used, a tissue known to contain its corresponding antigen should be included in every trial, so that staining intensity can be monitored for consistency. Sections where the primary antibody is omitted should also be included in each run, to reveal any non-specific staining by reagents used to detect the primary antibody. This latter control is unnecessary when the direct method is being used.

3. HYPOTHESIS AND OBJECTIVES

3.1 Project Hypothesis

It was hypothesized that thymic myoid cells are potential muscle precursor cells (mpcs). As presumptive mpcs, myoid cells should respond to an acute demand for new muscle cells, such as after a crush injury. The response might consist of changes in density and distribution in the thymus, able to be detected by *in situ* hybridisation. It was further proposed that the response would differ between *mdx* and control mice due to the differing requirements for mpcs that exist between the 2 strains.

3.2 Specific Objectives

1. To determine whether myoid cells can be identified by non-radioactive *in situ* hybridisation using a digoxigenin-labelled probe for myogenin.
2. To characterize any pattern and density of myoid cells evident in *mdx* and control mice.
3. To ascertain the myoid cell response, if any, after a crush injury to the right tibialis anterior (TA) in both normal and dystrophic mice and describe any differences observed between the two strains.
4. To learn whether myoid cells are undergoing proliferation as a possible consequence of the greater need for mpcs after a crush injury.

4. METHODS

4.1 ANIMALS

Colonies of Swiss Webster (SW) and *mdx* mice (Bulfield *et al.*, 1984) were housed in the Central Animal Care complex at the University of Manitoba and maintained according to the Canadian Council on Animal Care. The experiments were carried out with animal protocol approval from the University of Manitoba Animal Care Committee. SW mice were used in lieu of the C57Bl/10ScSn mice, the true control strain for *mdx* mice, because new inbred colonies were in the process of being established. As C57Bl/10ScSn animals became available, they were used in other segments of the project. In the subsequent sections, the term "control", with regards to mouse tissue, will be in reference to normal tissue (either C57Bl/10ScSn or SW) as opposed to *mdx* dystrophic tissue.

A total of 38 mice (19 SW; 19 *mdx*) were used in the experiment. Litters of 4-9 weanlings from each strain were randomly assigned to one of 3 treatment groups: those that received a crush injury, either 2 or 10 days before sacrifice, and those that served as untreated controls (no crush injury). Therefore, the group sizes (which included males and females) were as follows: control uncrushed (C0) n=6, control 2 day recovery (C2) n=5, control 10 day recovery (C10) n=8, *mdx* uncrushed (M0) n=4, *mdx* 2 day recovery (M2) n=9, and *mdx* 10 day recovery (M10) n=6.

It should be noted that thymus age, rather than age of the muscle, was standardized at the time of crush injury (though both would provide valuable information). This was to control for any developmental changes, such as in cell type complement, that the thymus might experience during growth.

4.2 CRUSH SURGERY AND TISSUE COLLECTION

Crush injury was delivered using a method (McIntosh *et al.*, 1994) modified after McGeachie and Grounds (1987). Mice were anesthetized with a 1:1 mixture of 100mg/mL Ketamine (final concentration = 1.5mg/mL) and 20mg/mL Xylazine (final concentration = 0.3mg/mL) administered at 1.5μL/g body weight. A small skin incision was made on the right leg of a mouse to expose the TA muscle, and the

fascia was opened. After separating TA from the underlying muscle bed, a hemostat was clamped (1 click) onto the belly of the TA muscle for 5 seconds and released. The wound was sutured closed and the mice were allowed to recover for the appropriate number of days. The contralateral leg of each mouse served as an additional control to the uncrushed control group. After the recovery period of 2 or 10 days, an intraperitoneal injection of tritiated thymidine (2μCi/g body weight) was given approximately 1 hour prior to sacrifice. Groups were killed by cervical dislocation under ether anesthesia at exactly 4 weeks-of-age. Each mouse was weighed and sexed.

Left and right TA muscle, thymus, and the jejunum of each mouse were removed. The wet weight of the thymus (cleaned of large blood vessels and fatty connective tissue) was recorded immediately upon excision. Tissues were embedded in OCT compound, and immersed in isopentane at -50°C. Cryostat sections cut at 5μm were collected on silanated slides (Henderson, 1989; see Appendix A). Sections were stored at -20°C until required.

A total of 86 mice (26 C57; 19 SW and 41 *mdx*) were used in the weight study (for a breakdown of the numbers based on gender see Tables 1 and 2). Values for the other strains were obtained as described above. Thymus and body weight values were imported into a Lotus 123 spreadsheet for calculation of thymus to body weight ratio for each mouse. Data were exported to the NWA Statpak program for statistical testing (Northwest Analytical Inc., Portland ORE).

4.3 *IN SITU* HYBRIDISATION (ISH)

4.3.1 Digoxigenin *In Situ* Hybridisation Protocol

Tissue sections were processed by fixation, hybridisation and visualization steps (modified after Garrett and Anderson, 1995; Figure 1A) and with appropriate negative controls as given below.

Sections were warmed to room temperature, fixed in 4% paraformaldehyde in 1xPBS for 15 minutes, followed by 2 washes in 1xPBS for 10 minutes. Unless otherwise stated, steps were performed at room temperature. Procedural control sections received either DNase (50U/mL in 40mM Tris buffer (pH 7.5) with 6mM $MgCl_2$) or RNase (50μg/mL in 0.01M sodium acetate (pH 5.2), 0.1M Tris buffer (pH 7.4))

treatments at 37°C for 15 minutes under coverslips. Later trials also included negative controls as follows: omission of anti-digoxigenin-alkaline phosphatase (α-DIG-AP) conjugate; omission of probe with α-DIG-AP conjugate; and omission of both probe and α-DIG-AP conjugate. Coverslips were removed and the sections washed well in millipore water and then in diethyl pyrocarbonate (DEPC) water. Hybridisation cocktail was boiled for 10 minutes then quenched on ice. Sections were prehybridised with at least 20μL of the cocktail under coverslips.

After a prehybridisation incubation of 1 hour at 42°C, sections were washed in DEPC water. Myogenin probe (labelled with DIG from Boehringer Mannheim, Germany) was boiled with hybridisation cocktail for 10 minutes at 95°C and cooled quickly (quenched) on ice. The sections were covered with 20μL of probe+cocktail solution, coverslipped, taking care to avoid air bubbles, and sealed with rubber cement. Hybridisation occurred overnight at 42°C in a humid incubator.

The next morning, coverslips were removed and the following washes occurred in large coplin dishes: 2 changes of 2x standard saline citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) in DEPC water for 15 minutes each, and 2 changes of 0.1xSSC, 0.1% SDS at 37°C. All washes were done with vigorous shaking (agitation). Sections were washed in DIG Buffer 1 (Boehringer Mannheim, components in Appendix B), blocked in DIG Buffer 2 (60 min.) then rinsed in DIG Buffer 1 (1 min.). The antibody conjugate (either DIG-AP or digoxigenin-beta-galactosidase (DIG-ß-Gal), Boehringer Mannheim) was diluted (1:500 and 1:100, respectively) in DIG Buffer 2, applied to sections, coverslipped and incubated overnight at 4°C. Sections were washed in 2 changes of DIG Buffer 1 with vigorous agitation, for 15 minutes each and equilibrated in DIG Buffer 3. Colour solution (9.0μL nitroblue tetrazolium salt [final concentration = 0.35mg/mL], 7.0μL 5-bromo-4-chloro-3-indolylphosphate [final concentration = 0.175mg/mL], and 20μL 24mg/mL levamisole in 2 mL Buffer 3) was applied to the sections and allowed to develop in the dark (foil protection is required as this solution is light-sensitive). At the appearance of colour precipitate (>14hr), a DIG Buffer 4 wash was used to stop the colour reaction. Sections were briefly stained by a few quick dips in hematoxylin and left to blue in running tap water (5 min.). Sections were not exposed to acid alcohol or dehydrating alcohols, as these removed the colour precipitate (positive

signal). Aquamount was used to mount the coverslips on sections. Results were recorded through photography on an Olympus BHT-2 photomicroscope equipped with bright field, phase contrast and epifluorescence optics.

4.3.2 Radioactive *In Situ* Hybridisation Protocol

The radioactive ISH protocol was the same as the DIG protocol with the following modifications. First, an acetylation step (0.09M triethanolamine with acetic anhydride, 0.25% (v/v) applied to sections for 10 minutes at room temperature and rinsed with DEPC water) was performed prior to prehybridisation. Sections were hybridised overnight with ^{35}S-labelled myogenin probe at 50°C, then dehydrated in 70%, 95% and absolute ethanol (5 min. each) and air dried at 4°C. Coverslips were removed in a 2xSSC wash, followed by washing in 4 changes of 2xSSC, 0.1% Triton X-100, 1mL ethylenediaminetetraacetic acid (EDTA) and 5μM dithiothreitol (DTT), one wash in 0.1xSSC, 0.1% Triton X-100, 1mL EDTA and 5μM DTT for 30 minutes at 60°C, and a wash in 2xSSC for 10 minutes at 60°C, all with shaking. Negative controls were sections incubated with DNase, RNase or sections which were not incubated with the ^{35}S-labelled myogenin probe.

After vigorous washing in 2xSSC, 0.1% SDS solutions, sections were rinsed in 1xPBS and ringed with a Dako pen for RNase A treatment for 40 minutes at 37°C. Sections were then washed twice in 2xSSC for 15 minutes at 60°C followed by a 1xPBS rinse and dehydration in a graded series of alcohols and left to air dry prior to autoradiography (see section 4.9). Autoradiograms were developed in D-19 (1:10 dilution of stock prepared according to manufacturer's directions) for 1 minute, rinsed in distilled water for 1 minute, and fixed (Kodak Rapid Fixer) for 2½ minutes at room temperature. Finally, sections were placed in hematoxylin (for RAG) for 4 minutes, rinsed in tap water and mounted with Aquamount.

4.3.3 Levamisole Titration of Thymic AP Activity

To determine whether higher concentrations of levamisole would inhibit the endogenous activity of thymic alkaline phosphatase, sections of thymus were prepared 3 separate ways to test whether AP activity was decreased at a specific concentration or when reaction occurred at specific intervals in the

procedure. Steps were carried out at room temperature.

The first set of sections was warmed to room temperature and labelled. The sections were fixed in filtered 4% paraformaldehyde in 1xPBS for 15 minutes and washed twice in 1xPBS for 10 minutes each. Sections were ringed with a Dako pen and levamisole was applied in various concentrations (0, 1mM, 10mM, 50mM, 100mM, 500mM, and 1M) for 30 minutes (a stock solution of 1M levamisole in DIG Buffer 3 was diluted to the required concentrations). Sections were then washed in 2 changes of DEPC water, dehydrated in a graded series of ethanol (70%, 95%, absolute) and air dried. Hybridisation cocktail (boiled and quenched) was added in 20-30μL quantities under coverslips, and incubated overnight at 45°C.

Coverslips were removed in 2 changes of 2xSSC, 0.1% SDS, for 15 minutes each, followed by 2 changes of 0.1xSSC, 0.1% SDS at 37°C for 15 minutes each. All washes were performed with shaking.

Sections were processed through the ISH procedures and the colour detection steps, as before (section 4.3.1). Colour precipitate was allowed to develop in the dark (3-27 hrs) during periodic monitoring under the microscope. When colour appeared, sections were rinsed and mounted with Aquamount.

The second set of sections was treated as the first set, with the exception that at the outset there was only a wash in 1xPBS, to remove the OCT compound, but no fixation step. Once dehydrated and dried, sections were placed at -20°C overnight. The following day, sections were allowed to warm to room temperature and processed as outlined above.

A third set of sections was warmed to room temperature and proceeded directly to the colour detection step (no pretreatments). The concentration of levamisole contained in the colour solution was varied according to concentrations stated above and applied to the sections.

4.4 TRANSMISSION ELECTRON MICROSCOPY (TEM)

4.4.1 Detection of Alkaline Phosphatase Activity

The method for detecting AP activity in thick frozen sections was modified after Hugon and Borgers (1966). Thymus was removed from one 8 week old C57Bl/10ScSn mouse and one 10 week old *mdx* mouse, embedded in OCT compound and snap frozen in isopentane at -50°C. Gut tissue served as a

positive control for the TEM detection of AP-activity. Frozen sections were cut to 15μm thickness and placed in 1xPBS to remove the OCT compound. Sections were fixed (2 hours) in 3% glutaraldehyde in 0.1M Sorenson's phosphate buffer and left overnight at 4°C in a solution of 5% sucrose in 0.1M Sorenson's buffer. Sections were washed briefly in 0.2M Gomori's tris-maleate buffer (pH 8.2) then incubated for 10 minutes in 20 mL of a freshly prepared medium consisting of 0.2M Gomori's tris-maleate buffer (2mL), 1.25% sodium glycerophosphate (4mL) and 1% lead nitrate (2.6mL) in ddH_2O to deposit an insoluble salt at the site of AP activity. (The solution was heated to 37°C for 15 minutes, cooled to room temperature and filtered prior to use.) After incubation, sections were washed in a 5% ammonium sulfide solution (stock concentration=20% $(NH_4)_2S$.Aq) for 2 minutes, fixed for 1 hour in 0.1% osmium tetroxide in Sorenson's buffer, dehydrated in a series of alcohols (10 minutes each in 30, 50, 70, 95, 95, 100, 100, 100% ethanol and 20 minutes in 100% methanol), infiltrated and embedded in epon resin. Washes were performed in multiwell tissue culture plates (Falcon 3047). Sections were then transferred to molds, allowed to settle for >24 hours in pure plastic, and baked at 60°C for 1-3 days. Thick sections were cut on a Reichert OmU2 ultramicrotome (P. Perumal) and stained with toluidine blue for orientation. Thin sections (pale gold interference colour) were collected on copper grids and stained with uranyl acetate and lead citrate. Sections were viewed and photographed on a Philips 201 electron microscope.

4.5 IMMUNOHISTOCHEMISTRY (IHC)

Immunohistochemistry on frozen sections was used to detect thymic cells which contain muscle-specific proteins. Sections stored at -20°C were allowed to warm to room temperature and dry for 1 hour. Sections of interest were outlined with a DAKO pen and processed exactly as reported (Anderson *et al.*, 1991). Antibodies (and dilution) included anti-troponin T (1:200), anti-skeletal myosin (1:20), anti-desmin (1:20)(Sigma ImmunoChemicals), anti-skeletal actin (1:50)(Amersham), anti-developmental myosin heavy chain (1:50)(Novocastra Laboratories Ltd) and anti-MyoD (1:100)(gift from Dr. P. Houghton, Memphis). The monoclonal antibodies (to troponin T, actin, developmental myosin heavy chain, MyoD) were raised in mouse, while polyclonal antibodies (to skeletal myosin, desmin) were raised in rabbit. The appropriate

biotinylated secondary antibody (anti-mouse or anti-rabbit, respectively) was applied to sections for 2 hours at room temperature, sections were rinsed in PBS, and incubated in Texas Red-streptavidin (30 minutes, at 1:250). Sections serving as controls for the procedure did not receive any primary antibody. Fluorescence was observed and photographed under an Olympus BHT-2 microscope equipped with epifluorescence optics.

4.6 MYOID CELL COUNTS AND AREA MEASUREMENTS

Sections stained by immunohistochemistry were coded by another member of the lab and each slide was examined with the microscope. TA muscle sections on the same slide served as positive controls for the IHC procedure. Muscle sections from positive and negative procedures were compared: bright, glittery red fluorescence in the location appropriate to myosin, actin and troponin in sarcomeres was interpreted as positive specific staining. Dull red fluorescence was considered negative. If sections incubated without primary antibody were negative for fluorescence, thymus sections on the same slide were examined for myoid cells (cells fluorescent after IHC staining for troponin T, i.e. troponin-positive). An ocular grid (each side of an individual square = 19.0μm at 400x) was used to determine whether a positive cell was large (9.5≥μm) or small (<9.5μm). The number of large and small myoid cells were recorded in each section, and the area of each thymus section was measured.

Area measurements were obtained by making paper tracings of each thymus section using the Nikon ALPHAPHOT YS Camera Lucida microscope at 100x power. Drawings were retraced on a calibrated graphics tablet using the SigmaScan Scientific Measurement System program (Jandel Scientific). Data were imported into a Lotus 123 spreadsheet, and compiled with cell counts, for calculation of cell density (# per mm^2) for each animal (total of 3-5 sections per mouse). The normalized cell counts will be referred to as myoid cell density. Data were exported to the Statpak program for statistical testing. Serial sections were prepared for autoradiography (see section 4.9 below) to detect DNA synthesis, and counterstained with Gomori trichrome stain as previously applied in this laboratory (McIntosh and Anderson, 1995).

4.7 STATISTICAL ANALYSIS

Three-way analysis of variance (ANOVA) was used to analyze the data. The three variables were mouse strain (normal or *mdx*), treatment group (no crush and crush with either 2 or 10 day recovery) and myoid cell density (large, small or total, each normalized to the area of thymus examined). Duncan's multiple range tests were performed if the 3-way ANOVA was significant. A probability of $p \leq 0.05$ was considered significant.

4.8 MICROSCOPY AND PHOTOGRAPHY

Photographs were taken with an Olympus C-35AD-4 camera mounted on an Olympus BHT-2 RFCA light microscope (LM), and using a Philips 201 transmission electron microscope (TEM). For LM, 400 ASA film was wound onto a spool in complete darkness, then processed in T-max developer for 7 minutes, indicator stop bath for 2 minutes, rapid fixer for 5 minutes, (Kodak, agitation every 30 seconds), cold running water for 20-30 minutes, Photo-Flo 200 solution for 30 seconds and dried. Similarly for TEM, film was wound onto a spool and processed with D-19 developer for 2 minutes followed by indicator stop bath for 30 seconds, rapid fixer (Kodak) for 3 minutes, washed (20 minutes in cold running water) and dried.

4.9 AUTORADIOGRAPHY

In darkness (red safelight), slides were dipped in K-5 emulsion, melted in a waterbath at 42°C and dried slowly in a humid oven (minimum 2 hours). The dried slides and desiccant were placed in black slide boxes made light-tight with tape, boxed and exposed at 4°C for 10 days (ISH with ^{35}S-probes) or 7 weeks (^{3}H-treated animals to detect DNA synthesis).

5. RESULTS

5.1 THYMUS WEIGHT

The body weight of many mice was measured prior to any dissection. The weight of the thymus was recorded immediately after its dissection from each mouse, to determine whether any differences existed among the strains used (26 C57Bl/10ScSn, 19 Swiss Webster (SW), and 41 *mdx*) and what impact the size of the thymus might have on the number of myoid cells found. The thymus weight tended to be higher in females compared to males of all strains (approximately equal numbers in each gender, see Table 1), although this was only significant in the Swiss Webster strain ($p<0.01$, Duncan's). The SW strain (both sexes) had significantly heavier thymus glands than the control strain ($p<0.01$, Duncan's for males and females). Thymus weight was also significantly greater in SW mice (both genders) when compared to the *mdx* strain ($p<0.01$ for males, $p<0.05$ for females, Duncan's). The weight of control and *mdx* thymuses did not differ significantly. When thymus weight was normalized to body weight (Table 2), significant differences ($p<0.01$, Duncan's) were found only when comparing the female SW mice to the other two strains (both sexes). SW females still had significantly heavier ($p<0.01$, Duncan's) thymuses than their male counterparts. After normalization to body weight, thymus weight was found to be significantly lower ($p<0.0001$, 1-way ANOVA) after a muscle crush injury was imposed. The decrease in normalized thymus weight differed significantly ($p<0.0001$, 1-way ANOVA) among the 3 strains, the greatest decline being observed in *mdx* mice and dropping the least in C57 mice (see Appendix C). The response of thymus weight (normalized to body weight) to the crushed and uncrushed status of the right TA muscle was also significantly different between C57, SW and *mdx* strains (interaction effect, $p<0.01$).

5.2 *IN SITU* HYBRIDISATION (ISH)

In situ hybridisation was used to determine whether cells expressing the muscle-specific regulatory factor myogenin (myoid cells) were present in the thymus, and if so, their distribution in the tissue. Probes to myogenin, a regulatory transcription factor specific to skeletal muscle, were labelled with digoxigenin

(DIG) or ^{35}S. In total, 3 experiments using DIG-labelled probes and 4 experiments with radiolabelled probes were conducted.

5.2.1 Muscle ISH and Procedural Controls

In the non-radioactive trials with DIG, the positive signal was a dark brown stain or precipitate, and was observed on both muscle and thymus tissue. The signal was localized to the cytoplasm of newly formed myotubes in dystrophic areas of *mdx* TA muscle (Figure 1B). Only the occasional mononuclear cell was myogenin-mRNA positive in C57Bl/10ScSn TA muscle. The sections treated with DNase yielded similar results (not shown), while the RNase incubation of the procedure on muscle tissue removed all staining for mRNA (Figure 1C). Therefore, the ISH procedure was specific on muscle sections.

5.2.2 Thymus ISH

Thymus tissue from both mouse strains demonstrated positively-stained cells in the medullary centre on slides processed through the full ISH procedure (Figure 2A) and after DNase treatment (not shown). However, a similar distribution pattern and number of myogenin-positive cells was also present in all thymus sections treated as negative controls (RNase-treated, no probe, no anti-DIG-AP, and no probe/no α-DIG-AP) (Figure 2B-E). Thus, in contrast to muscle, in the thymus ISH-positive cells were not positive specifically by the mRNA detection of myogenin; that is, a false positive signal was always produced in DIG-ISH and was not distinguishable from the true positive signal using standard controls.

5.2.3 Alkaline Phosphatase

Further investigation revealed that the alkaline phosphatase component of the colour detection step was the cause of the false positive signal. Even in the absence of probe or when treated with RNase (Figure 2B), the ISH signal was unchanged from that produced by the full ISH process (Figure 2A). In previous ISH trials in the laboratory that involved only the colour detection step (that is, no probe or DIG-antibody) there were some vessels in the thymus that were labelled (Figure 2F). Although levamisole typically decreases endogenous AP activity, even very high levamisole concentrations did not obviously reduce thymic AP activity (Figure 2G), although it did appear to diminish the number of cells showing signal. Attempts with non-AP-conjugated antibodies to DIG, including digoxigenin-horse radish peroxidase (DIG-

POD), and digoxigenin-beta-galactosidase (DIG-ß-Gal), did not produce any signal in the thymus in 2 separate experiments with each detection system. Since endogenous alkaline phosphatase activity in the thymus could not be eliminated, and attempts using other enzyme-conjugated antibodies were unsuccessful, the non-radioactive ISH procedure was abandoned.

5.2.4 Radiolabelled Riboprobes

ISH with radioactively-labelled myogenin probes revealed areas of cells that were labelled with exposed silver grains in both TA muscle and thymus. Silver grains in concentrations higher than background levels, indicative of a positive signal, could be seen overlying myotubes and in the surrounding areas of active inflammation from dystrophy. In the thymus, dense collections of grains covered groups of cells and single entire cells. Positive cells in the thymus were located primarily in the medulla (Figure 3A), covering areas upwards of 50µm in diameter, and appeared to be more common in control than *mdx* animals. DNase-treated sections were observed to have a similar deposition of silver grains (Figure 3B, inset). Sections processed by RNase treatment had a lower density of silver grains over cells in the thymus compared to those without RNase treatment after the same exposure time. However, after longer exposure, the autoradiograms did relatively closely resemble the positive control slides which received the full ISH procedure (Figure 3C,3D). Background silver grains were very low in number in the absence of probe (Figure 3D, inset), so contamination was not the source of the false positive signal in the radioactive ISH process with RNase. However, identification of positive cells was not possible, since silver grains obscured the underlying cell features and often appeared to extend over more than one cell. The obstruction of cellular detail also meant that any attempt to determine myoid cell number would be invalid using radiolabelled ISH probes, as multiple positive cells in close proximity to one another would not be distinguishable from a solitary positive cell. Therefore, radioactive *in situ* hybridisation was not pursued.

5.3 ELECTRON MICROSCOPY (EM)

Electron microscopy was used to visualize alkaline phosphatase activity in the gut and thymus in

order to determine whether myoid cells were the origin of the false positive signal by DIG-AP ISH with RNase or high levamisole concentrations. (Counting myoid cells by EM would be predicted to be a very time-consuming task.) Gut was used as a positive control since it is known to have a high AP activity (Gomori, 1939). The quality of the EM sections did not show good preservation of ultrastructure because the tissues were not preserved first before freezing and sectioning (see section 4.4.1). There were darkly stained areas on the surface of epithelial cells facing the gut lumen (Figure 4A), over the membranes (Figure 4A, inset) and glycocalyx of the brush border (Figure 4B) and within the cell, indicating the typical localization of AP activity.

General characteristics of the thymus (Figure 5A) were comparable to previous ultrastructural studies (Alvarez, 1990). Apoptotic lymphocytes were quite striking and abundant (Figure 5A, arrows) throughout the uniformly staining thymic tissue. Myoid cells were identified by the presence of myofibrillar elements in the cytoplasm, which often tapered into processes (Figure 6A). Myofibrils could be distinguished from collagen fibrils outside the cells on the basis of location and the intensity of staining (Figure 4C,4D). Six non-myoid cells were observed to have AP activity, which appeared as dark periodicities just inside the plasma membrane (Figure 5B,5C). Cells containing the periodicities are thought to be interdigitating cells (identified by characteristic multiple processes; Alvarez, 1990), but dense periodic staining was absent from myoid cells. The periodicities did not appear in sections of thymic tissue which did not receive substrate as negative procedural controls. Thymus sections not fixed with osmium had poor preservation that prevented good cell identification, but some areas of dense staining (like the AP deposit) were noted in the tissue.

5.4 IMMUNOHISTOCHEMISTRY (IHC)

Antibodies to troponin T, skeletal myosin, desmin, skeletal actin, developmental myosin heavy chain and MyoD were used initially as a screen to identify muscle-like myoid cells in the thymus. As a positive control, TA muscle sections were also placed on the slides. Only staining with antibodies to troponin T was found to be specific in showing a consistent appropriate sarcomeric pattern (Figure 5B)

which was absent in negative controls (no primary antibody) of muscle sections. Brilliant red fluorescence could be seen running along the length of the muscle fibers (Figure 5A); the bright fluorescent quality was entirely absent in TA muscles which did not receive primary antibody (Figure 5C). Similarly in the thymus, α-troponin T staining showed a localized intense cellular fluorescence that was absent in the absence of primary antibody. Cells positive for troponin in the thymus were considered to be myoid cells (Meinl, 1991).

Myoid cells varied in shape, from abundant oval or round cells to the less frequent polymorphous cells. Staining was homogeneous over most of a cell, and was at times very intense. In some cases, processes of variable length (up to approximately 10μm), extended from troponin-positive myoid cells (Figure 7B,C). The diameter of a process was roughly about 2-3μm. The size of the positive cells also varied, so cells were recorded as either large or small, as previously defined (section 4.6). Occasionally, cells that fluoresced in a broad ring were observed around blood vessels, likely smooth muscle cells (Figure 7F).

After immunohistochemical staining of the complete set of slides, large and small myoid cells were counted on the 2-4 sections of thymus on each coded slide. Careful records were kept for each section examined such that the number and type of myoid cell could be traced back to an exact section and its area, to be used in later analysis.

Myoid cell density was recorded for each thymus section examined. Slide numbers were then decoded, and data were analyzed on a spread sheet. The mean ± standard error of the mean (SEM) were determined for each group (data presented in Table 3). A 3-way ANOVA of the data revealed that SW thymus had a significantly larger ($p<0.0001$) myoid cell density than observed in the *mdx* mouse thymus. The type of myoid cell present also differed significantly between strains ($p<0.0001$, ANOVA) overall, small myoid cells were more dense than large cells in the thymus of both strains.

The response of large myoid cell density to recovery time (0, 2 and 10 days) also differed significantly (interaction effect, $p<0.01$) between the 2 strains (Figure 8): large myoid cells tended to decrease by approximately 33% (but not significantly) between 0 and 2 days recovery after crush, and

appeared to level off by day 10 post-crush in control animals. By contrast, in *mdx* mice the population density of large myoid cells rose slightly between 0 and 2 days recovery and then dropped (though not significantly) almost 78% by day 10 of recovery to levels below the original level in mice without muscle crush. Large myoid cell density at the initial and final time points were found not to differ significantly from one another in each of the mouse strains. However, in pairwise comparisons, the observed strain differences were significant ($p<0.01$, Duncan's) at each of the 3 recovery time points examined.

The number of small myoid cells normalized to area also differed significantly ($p<0.05$, Duncan's) between the 2 strains, although only in mice which did not receive a crush injury, suggesting that small cells positive for troponin T are different from large troponin-positive cells. The apparent changes in the density of total myoid cells reflect changes in the large cells but were not significant, likely due to the combination with the small cell data that showed no significant changes.

5.5 AUTORADIOGRAPHY

Consecutive sections of muscle and thymus were taken through the autoradiography procedure and counterstained by Gomori's trichrome method to determine if any of the myoid cells were synthesizing DNA in preparation for proliferation. Positive cells were seen under high magnification (cells with 20 grains or more) and were found in the muscle within small myotubes (Figure 9A) and in the thymus as mononuclear cells (Figure 9B). However, the high density of small cells in the thymus (within a 5μm thick section) and the inability to determine whether the dividing cells were myoid cells by conventional staining, made the autoradiography of Gomori-stained sections non-contributory in addressing the question of myoid cell proliferation. Therefore, the complete set of Texas-Red fluorescent IHC slides were dipped and processed for autoradiography after all cell counts and morphometry were completed. Roughly one out of every twenty myoid cells, as identified by IHC, were found with 3-8 overlying silver grains (not shown). Thus, this portion of the study has shown that although TnT-positive cells can be dividing, the majority are not.

6. DISCUSSION

6.1 OVERVIEW OF RESULTS

The results of this study indicate that *mdx* and control mice have different numbers of myoid cells in the thymus and that the myoid cells of the 2 strains respond differently after crush injury. Swiss Webster mice were used as controls for this study because colonies of the proper control strain for the *mdx* mouse (C57Bl/10ScSn) were unavailable for use when required. Thymus weight was investigated and observed to have a possible relationship with myoid cell population in a given strain.

Non-radioactive *in situ* hybridisation (ISH) was not specific for myoid cells, and instead also stained blood vessels and epithelial cells. The appearance of positively-labelled cells in negative controls prompted further investigation of the ISH protocol and revealed that levamisole-resistant alkaline phosphatase (AP) activity is endogenous to the thymus. Taken together with electron microscopy findings that showed myoid cells and AP-expressing cells were two distinct populations within the thymus, changes were made to ISH detection step. Instead of using the AP-linked detection of digoxigenin (DIG), substitutions were made with beta-galactosidase and horse radish peroxidase. However, neither was sensitive enough to detect myogenin in myoid cells. Thus, neither myoid cell density nor distribution could be ascertained by DIG-ISH.

Using an antibody to troponin T, myoid cell density was found to be significantly greater in Swiss Webster (SW) than *mdx* mice. Crush injury caused different reactions in the thymus of the 2 strains.

6.2 TECHNICAL ASPECTS

Initial attempts to identify myoid cells were made by *in situ* hybridisation, which is able to detect the mRNA of the early skeletal muscle marker, myogenin, in very small amounts. Using a radioactive technique, thymic cells have been shown to contain transcripts of MyoD and myogenin (Grounds *et al.*, 1992a). It was hoped that a non-radioactive method could be found so that silver grains would no longer interfere with the observation of cellular features. Although the non-radioactive trials with DIG-labelled

probes were specific for muscle tissue mRNA for myogenin, the procedure could not be used for the thymus because the alkaline phosphatase-linked antibody in the detection step led to the appearance of positive cells on negative control slides. High concentrations of levamisole at various stages throughout the ISH procedure were unsuccessful at removing the endogenous AP activity of the thymus. Gomori (1939) reported muscle as having either trace amounts or no phosphatase at all. He further cited that capillary endothelium as well as the epithelium and connective tissue of mammillary glands contained some of the highest levels of AP activity in the body. It seems possible (from EM studies and previous reports) that the epithelial elements of the thymus, including interdigitating and epithelioreticular cells, might be expressing AP activity, in addition to the pervading vasculature of the thymus. Eosinophils, a type of white blood cell that is resistant to conventional means of suppressing AP activity, do have access to the thymus through the blood vessels and may also contribute to the observed false positives. EM analysis suggested that myoid cells and alkaline phosphatase-expressing cells were two discrete populations, so misleading numbers would likely be determined for either population by the use of an AP-linked detection system.

Several ISH trials were attempted with DIG antibodies conjugated to enzymes other than AP. DIG-ß-Gal (2 experiments) and DIG-POD (4 experiments) did not detect the presence of any myoid cells in the thymus, possibly because optimal experimental conditions for the DIG-ß-Gal system (such as buffer concentrations, temperature, pH) still had to be empirically determined, while the latter system was not sensitive enough. Northern analyses were also unable to detect myogenin in the thymus in another study in the laboratory (K. Garrett, unpublished data). This suggests either that myogenin mRNA in the thymus is such a low level that only the most sensitive detection systems (such as reverse transcriptase-polymerase chain reaction or RT-PCR) will demonstrate it, or that there is no myogenin mRNA in SW, C57Bl/10ScSn or *mdx* thymus (contrary to the study by Grounds *et al.*, 1992a, which examined different strains of mice).

Previous studies using SJL/J and BALB/c mice have shown that the presence of myogenin mRNA in cells of the thymus can be detected by radioactive ISH (Grounds *et al.*, 1992a). Results were reproducible to an extent in our own lab. We observed a distribution of silver grains over cells in thymus sections similar to the previous report. However, unlike the previous study, we noted the pattern also arose

in the RNase-treated negative controls for the experiment. Even in the late stages of involution, the thymus is still active and producing functional lymphocytes. Perhaps lymphocytes or other thymic cells are dividing and the incorporation of tritiated thymidine, injected just prior to sacrifice, was exposing silver grains that could be mistaken for myogenin-positive cells. The latter possibility is remote, since the exposure time required by tritiated thymidine is considerably longer (6 weeks) than for ^{35}S (10 days).

The resolution of radiolabelled probes is also less than for non-radioactive probes. Silver grains obstructing the underlying cellular detail made it difficult to distinguish what type of cell was labelled for myogenin message. It was also questionable whether the technique could differentiate a single cell from an aggregation of cells, as non-radioactive probes typically do. Previous reports describe myoid cells as scattered singly throughout the medulla (Valikova *et al.*, 1982), in clusters (Henry, 1966; Drenkhahn *et al.*, 1978), or both (Van de Velde and Friedman, 1970; Puchtler *et al.*, 1975), the latter in agreement with observations from this study. Radiolabelled probes did not seem adequate for resolving single cells (Grounds *et al.*, 1992a) in order to test the current hypothesis. Thus, due to the limits of the procedure, the original question of determining myogenin-positive myoid cell number and distribution could not be answered and so the ISH protocol was abandoned as the principle approach for discerning myoid cells.

Myoid cells were, however, clearly identified with electron microscopy (EM) by the presence of myofilaments in the cytoplasm of cells larger than lymphocytes. It was hoped that an insoluble colour precipitate or silver grains would mark (by ISH) the location of myoid cells in light microscopy (LM). Of the 2 techniques, LM was preferred, as the odds of sectioning through a myoid cell for an EM section are low and would not be statistically valid unless a tremendous number of sections were systematically viewed.

In situ hybridisation and immunohistochemistry are equally sensitive detection techniques, differing in their targets of mRNA and protein, respectively. Sassoon *et al.* (1989) used ISH to detect myogenin mRNA in muscle, which appeared 2 days earlier than myogenin protein, detected by IHC. Myoid cells are rarely found in human thymuses beyond 7 months gestation (Henry, 1966), with the exception of those found in the abnormal conditions of the thymus (Müller-Hermelink and Marx, 1994). Since myoid cells have been observed arrested in all stages of development, and would not necessarily contain troponin T, then

detection by ISH would be preferable to IHC. Muscle regulatory factors are expressed in all skeletal muscle cells, and most likely also by myoid cells, but muscle-specific proteins may not be expressed for some time after myogenin, so there would be a greater likelihood of finding a larger proportion of myogenin mRNA-positive than troponin T-positive myoid cells.

6.3 THYMUS

The developmental stage of the thymus was standardized by killing the animals at 4 weeks-of-age. Despite this measure, intrinsic size differences were apparent among the mouse strains. Swiss Webster mice, which had significantly heavier thymus weights than C57Bl/10ScSn or *mdx*, also had the highest density of myoid cells, which suggests that increasing size might play a role in promoting the larger density of myoid cells in the thymus. It would be interesting to learn whether myoid cell density is also consistent with sex, since female mice are reported to have larger thymuses than males, an observation confirmed by the present study. However, once beyond the dissection stage, the sex of the mouse from which the thymus originated was unfortunately not recorded, and the number of mice was much smaller in the crush injury study than for the parameters of thymus weight. However, myoid cell density seems to be consistent regardless of physical size, even though body weight itself differs between C57Bl/10ScSn controls and *mdx* mice (Anderson *et al.*, 1987), and comparable results were found when examining thymus weight alone or thymus weight normalized to body weight.

The abundance of apoptotic cells observed in the thymus by EM was not unexpected, as the majority of lymphocytes developing there do not leave but rather are destroyed. The electron-dense periodicities seen in cells with the AP activity is probably due to the arrangement of cell surface receptors on the cytosolic side of the cell membrane. The identity of the AP-expressing cells, a group distinct from myoid cells, was not clearly ascertained because of poor preservation of cell morphology in the EM sections, although they are likely to be interdigitating cells according to the general morphology that was observed (Alvarez, 1990). The appearance of myofilaments loosely scattered in the cytoplasm of putative myoid cells in the present study, is in agreement with previous ultrastructural reports of myoid cells (Raviola and

Raviola, 1967). The tapering poles of myoid cells by EM are likely to be congruent with the processes that were often seen on myoid cells by IHC.

6.4 MYOID CELL VARIATION IN THE THYMUS

Myoid cells detected by the antibody to troponin T were categorized as either large or small (as defined in section 4.6). There was a significant interaction ($p<0.01$) between strain and treatment by large cell density. It is not known whether this response by the large myoid cells to injury and recovery was a primary or secondary change. Previous studies have reported myoid cells to be fewer in number and arrested at less developed stages in the adult than in younger animals (Raviola and Raviola, 1967). Even such small and varied populations are thought to be able to generate marked effects in the thymus, such as in myasthenia gravis, where myoid cells have been implicated in the etiology of the condition. The significant change in cell density in the current study was presumably in response to the immediate need for more cells to aid in the recovery of the crush-injured muscle. The myoid cell density changes were not thought to be the result of a defective immune system, as all 3 strains including *mdx* (Guérette *et al.*, 1995) are immunocompetent. Coupled with the myoid cell type's skeletal muscle properties (see section 2.2), the present results suggest the myoid cell population could act as muscle precursor cells and could be recruited for the muscle repair process. If indeed myoid cells are mpcs, one can speculate that there must be a balance between the formation, development, and maturation of the myoid population in the thymus, and myoid cell export from the thymus to muscle which will ultimately result in a population density at a particular time.

Demand would influence the balance: these changes would be in addition to the increased mpc requirement in crush-injured muscle compared to healthy, undamaged muscle. The continuous need for mpcs in muscle would be high in *mdx* mice due to dystrophy. However, demand would change more dramatically in normal mice after muscle injury than in *mdx* mice after injury. Myoid cell populations were found to be smaller in thymuses of dystrophic mice possibly due to the larger, and more continuous need for mpcs in *mdx* muscle. In control thymuses, myoid cells may be a larger population and act as a reservoir

of mpcs, since the need for mpcs is small without ongoing dystrophy.

McIntosh *et al.* (1994) characterized the events of muscle regeneration after crush injury in C57Bl/10ScSn and *mdx* mice. It was found that the peak need for mpcs occurred 2 days after muscle damage occurred and was essentially complete by day 10 post-injury. The observations prompted the decision to use corresponding time periods in the current study. In McIntosh and Anderson (1995) it was also shown that the time course is about half a day faster in *mdx* than C57Bl/10ScSn mice (this is not the case for the Swiss Webster strain, which has a comparable muscle regenerative capacity to the *mdx* mouse). A significant effect ($p<0.01$, Duncan's) was also found between the 2 strains with respect to recovery time for thymic myoid cells in the present study.

In controls, large myoid cells (possibly more mature than small cells, and ready for export) may be depleted from the thymus during the early stages of muscle regeneration when the need for mpcs in muscle is rising after crush injury. However, the depletion was not significant possibly due to wide variation in the group, and to the small number of cells present at day 0. The need for mpcs might be expected to be lower as the muscle repair process nears completion, and the rate of export could slow. From this study it cannot be determined whether small cells mature into large myoid cells, but if that is the case, then ongoing development into mature large cells could result in a plateau in the large cell population as demand continues. Thus, the difference in response by large and small cells could be due to the differential demands and population influence for each type, assuming maturation from one type of cell to the other.

In contrast, the trend in *mdx* thymus was for the density of large myoid cells to increase with crush injury, though not significantly. Uncrushed *mdx* muscle already has a requirement for mpcs, so a crush injury might increase demand enough to push the thymus beyond a threshold, to speed up the development of possibly immature, smaller myoid cells, and result in the tendency to rise, or more accurately a plateau in the number of large cells present. Further in this line of speculation, a possible explanation for the tendency for large myoid cell density to drop during the latter stage of regeneration is if demand for mpcs continued: demand might exceed the barely adequate supply, resulting in large myoid cells leaving the

thymus in greater numbers.

Near the end of muscle regeneration, the large myoid cell density in both strains was below the respective initial pre-crush levels (not significant). In control mice, the level was stable after 2 days post-injury, while the trend for the same population in *mdx* mice appeared to be still falling after 2 days. One possible explanation for the discrepancy is that the response of large myoid cells in *mdx* animals is both delayed and prolonged. The delay would allow time to maximize the pool of myoid cells (possibly augmented from rapidly maturing small cells). The prolonged repair period could result from the greater need for mpcs in *mdx* muscle which would not decrease to zero after 10 days as it would in control mice. This possibility could be confirmed by examining time points more than 10 days after injury to see if the large cell population stays stable (control) or becomes stable at the original level at some later time (*mdx*). It is not known whether a subsequent large demand for mpcs would re-initiate the same response pattern by the thymic myoid cells. It is also unclear whether the response could occur at the depressed levels of myoid cells recorded at 10 days after injury, or if the reserve of myoid cells would need to replenish prior to the re-commencement of the response. Indeed, one wonders if there is a limit to te capacity to respond, and if so, is that limit related to the age-related decline in regeneration capacity in mice (Zacharias and Anderson, 1991) and in human DMD muscle (Webster and Blau, 1990).

Therefore, it is possible that the differences observed in the response of thymic myoid cells between the 2 mouse strains are due to 2 responses at work, one resulting from repair timing (faster in *mdx* than controls) and the other a consequence of the general demand for mpcs (larger and continuous in *mdx* compared to control).

Small myoid cells may be more prevalent because larger ones have matured enough to be exported for muscle regeneration outside the thymus. Alternatively, the small cells may represent more frequently sectioned processes of larger cells or simply immature cells. In any case, it is not unexpected that significant differences were observed in the responses by small and large cells. Small myoid cell density was also significantly higher in control than dystrophic thymuses, but only in uninjured mice, very consistent with the differing mpc requirements in the 2 strains (discussed above). This sets small myoid

cells apart from the large, the density of which exhibited a significant strain difference at all 3 time points examined.

The quick response of myoid cells (2 days) to crush injury in both strains is consistent with a putative role as mpcs. This response is short-lived though, as numbers drop by 10 days post-crush. This is a possible indication that the extent of the thymic contribution of mpcs to myogenesis is quite limited in terms of absolute numbers supplied and the period in which they are provided.

Filamentous processes on myoid cells may provide mobility to myoid cells, a necessary component for any migration to the site of damaged muscle. Presumptive mpcs could also possess surface molecules, such as NCAM (for example, McIntosh and Anderson, 1995), which are attractive to muscle, to aid in migration to the area of need, similar to receptors on inflammatory cells (Adams *et al.*, 1994), and to aid fusion with other mpcs (Mege *et al.*, 1992) once they reached muscle.

Overall, the myoid cell population cannot be a huge primary contributor to muscle regeneration in the short term because the total numbers generated in the thymus would be small (a very crude estimate puts the total at approximately 425 in the 4-week-old thymus of Swiss mice and only 80 for a corresponding *mdx* thymus) compared to the number of mpcs required to repair a crush injury (McIntosh and Anderson, 1995; Mitchell *et al.*, 1992). It is possible that mpcs of thymic origin could be 'blast-like' enough to take up the satellite cell role on new myotubes or old fibers. Secondarily, if myoid cells have a greater proliferative capacity than myosatellite cells, the potential contribution to the repair process over the long term could be more substantial than the numbers first suggest. This was not the case, however, as determined from our autoradiography study. Results showed that of the many cells undergoing DNA synthesis, only a tiny fraction are myoid cells. Furthermore, the number of silver grains over dividing cells suggests that myoid cell division (with 3-8 grains) occurs at a slower rate than myoblasts (35 or more grains) found in muscles of the same animal.

The myoid cell responses in this investigation were attributed to the muscle crush injury imposed on the mice. However, the observed responses could be non-specific with respect to the tissue being crushed, that is, injury to another organ (such as the liver or kidney) might produce similar results. A future

study could address this concern by including such a control.

It is also plausible that the responses of myoid cells to crush injury could be an artifact of the mass exodus of lymphocytes, creating a perceived increase in myoid cell density when the population actually remained the same. The response of myoid cells was different between the two strains, but total numbers in the thymus would need to be determined by different means than employed in the present study.

Staining myoid cells with antibodies to troponin T only detected those myoid cells mature enough to express troponin T protein (late in the development of skeletal muscle). Previous studies have reported myoid cells arrested at many stages of development. Detection of muscle markers expressed earlier in muscle development, such as myogenic regulatory factors or other muscle-specific proteins like skeletal actin, would provide a more complete assessment of the number of myoid cells, although there might not necessarily be a response to crush injury by the more immature myoid cell population. Although extensive ISH trials were undertaken in the lab, not all avenues for the molecular biology technique were exhausted due to time restrictions. Immunohistochemistry may even be used to address this concern by staining for the protein to MyoD, for example, although a reliable commercial antibody has only just become available.

In addition to other probes to be used in ISH identification of myoid cells, negative controls can be further supplemented by sense probes and irrelevant labelled probes (for radioactive ISH in particular) to obtain more confident and more convincing results.

The question as to why the radioactive *in situ* hybridisation protocol did not work in our lab, but was successful elsewhere (Grounds *et al.*, 1992a) must be asked. The answer can be addressed from 2 aspects: the technique and the reagents. Although the method was strictly adhered to, it's possible that stringency, which was based on optimum levels for muscle tissue processed on the same slides, was not equally effective on lymphoid tissue. Perhaps mRNA is more stable at test conditions in thymus than previously anticipated in muscle, necessitating a longer incubation time with the RNase before all mRNA is broken down. Alternatively, differences in mouse strains used (SW, *mdx* and later C57Bl/10ScSn in our lab; primarily SJL/J and BALBc in Grounds *et al.*, 1992a) may have contributed to the discrepancies observed between the results of the 2 laboratories. Furthermore, the density of myoid cells may be

inherently different between strains, or even between genders. A higher prevalence ought to facilitate the detection process. Records were not kept on the gender of the individual mice used in this study but both sexes were used (only females were used by Grounds *et al.*, 1992a). In addition, if the labelling of probes was weaker in the other study, the appearance of silver grains over an RNase-treated control section would not be apparent. The figure in the previous report (Grounds *et al.*, 1992a) does show some clumps of silver grains in the RNase-treated thymus section, albeit at low magnification (their Figure 1F) similar to those which were deemed unacceptable in the present study. EM *in situ* hybridisation, although uncommon and arduous, could satisfactorily determine the identity of positive cells in the thymus.

6.5 FUTURE INVESTIGATION

Experiments in future could focus on better characterization of myoid cells, such as the origin, a daily total of cell density after a muscle crush, examination of additional identification methods, determination of the cell expansion status, and investigation of the fate of myoid cells (whether an exodus from the thymus occurs and if so their final destination). All of these questions would evaluate the source, proliferation status, response, population size, and application of thymic myoid cells as putative mpcs. The present study is the first to support that possibility by showing a quantitative response to experimental manipulation under diseased and normal conditions.

The current study could be repeated, crushing both TA muscles instead of only one for an even greater demand for mpcs, and include monitoring of cell density at 24 hour intervals for a more complete response curve, using C57Bl/10ScSn as normal controls. Furthermore, the data suggests that the focus should be on the large myoid cell type.

The use of a single antibody to detect myoid cells was limiting, probably identifying only a fraction of the existing myoid cells. Compared to a previous publication figure (Grounds *et al.*, 1992a) the estimate by other means would suggest greater numbers of myoid cells are present than found in the current study. Diversifying the methods for discerning myoid cells, using other antibodies specific for skeletal muscle (additional IHC), or probes for MyoD (another ISH) for instance, can potentially identify a greater

proportion of this group of cells.

Migration studies might determine whether myoid cells actually leave the thymus, and if they go to muscle, whether they proliferate there or fuse into myotubes. Myoid cells may leave the thymus with the first wave of inflammatory cells, reach the area of need and stimulate satellite cells to get involved in the repair process. Perhaps only the daughter cells of proliferating myoid cells fuse to form myotubes at the site of muscle damage, ensuring a continuous supply of new daughter cells to serve as a reservoir of mpcs in future.

Current research with kidney cells injected into the thymus has shown that subsequent kidney transplant is not rejected (Perico *et al.*, 1991). Theoretically then, it might be possible to inject muscle cells into the thymus prior to direct injection into muscle, to augment the limited success of myoblast transfer therapy. This hypothesis could be tested in control and *mdx* mice.

6.6 CONCLUSIONS

This study has demonstrated that myoid cells of the thymus may have a role in the regeneration of damaged muscle. Specifically, the study concludes that:

1. Myoid cells could not be detected by *in situ* hybridisation using DIG-labelled myogenin probes, but were clearly identified by immunohistochemistry with an antibody to troponin T.

2. Swiss Webster mice had significantly greater density of large myoid cells than *mdx* mice. The distribution pattern of the cells in the thymus was not established in this study. Fluorescent counterstaining was not performed so little detail of the background tissue could be used for orientation to thymic medulla or cortex.

3. The response of large myoid cells to the crush injury differed significantly ($p<0.01$, Duncan's) between the 2 strains examined. Myoid cell density dropped then appeared to level off in SW mice, while in *mdx* mice, the corresponding population rose slightly then dropped essentially to zero.

4. Autoradiography showed that myoid cells can be dividing after a crush injury, but most are not.

Therefore, this study has tested the hypothesis, albeit by different methods than first proposed, and supports the possibility that a change in the density of myoid cells occurs after crush injury and differs significantly between normal and dystrophic strains.

7. FIGURES AND TABLES

FIGURE 1:

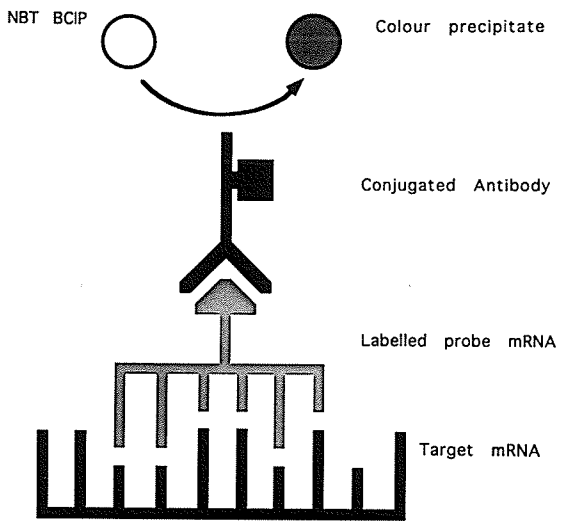
A: A schematic diagram of the components and steps involved in the technique of *in situ* hybridisation (see section 2.4).

Micrograph of longitudinal TA muscle section probed with digoxigenin-labelled riboprobe for myogenin. Arrows indicate a representative myotube (x240).

B: Positive signal in the cytoplasm of new myotubes with central nucleation. The muscle originated from an *mdx* mouse and these myotubes are in an area of dystrophy.

C: Myogenin mRNA staining is absent in the consecutive section of muscle treated with RNase.

In Situ Hybridisation Strategy



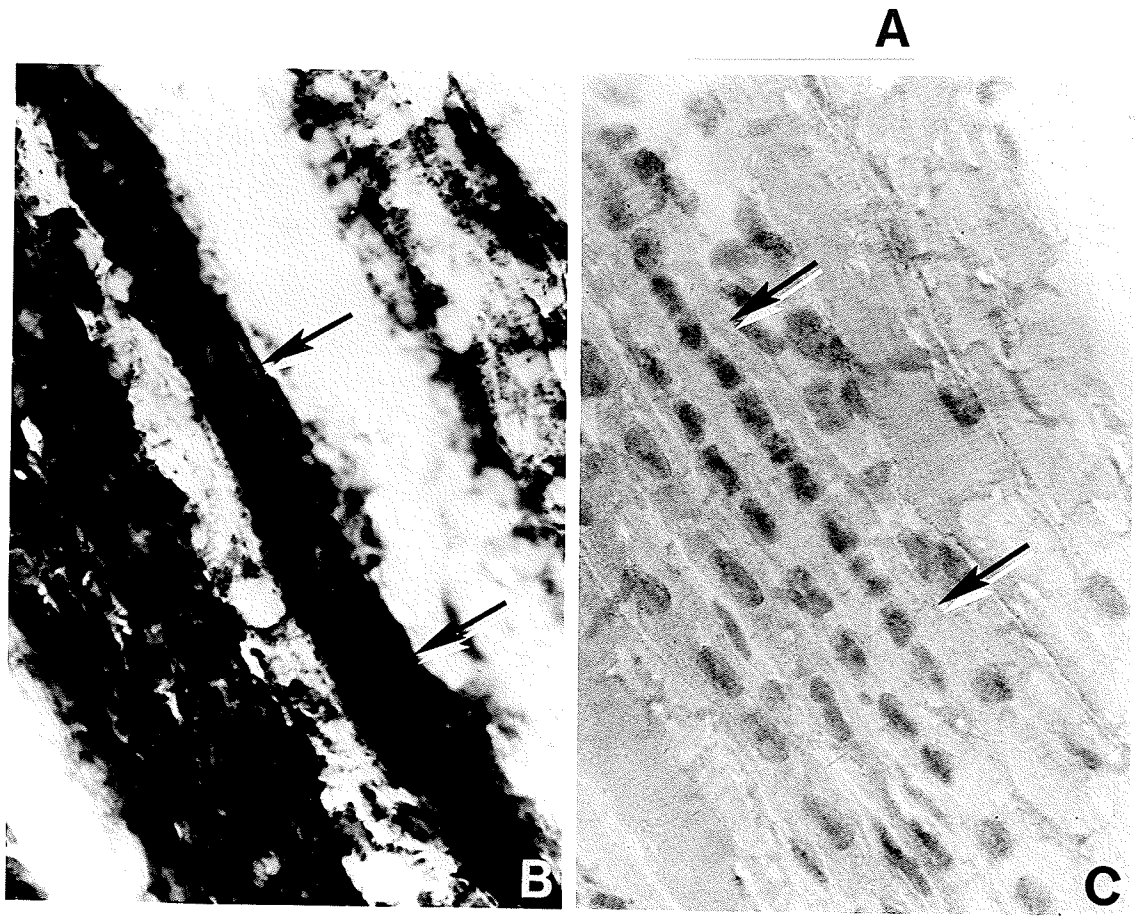


FIGURE 2:

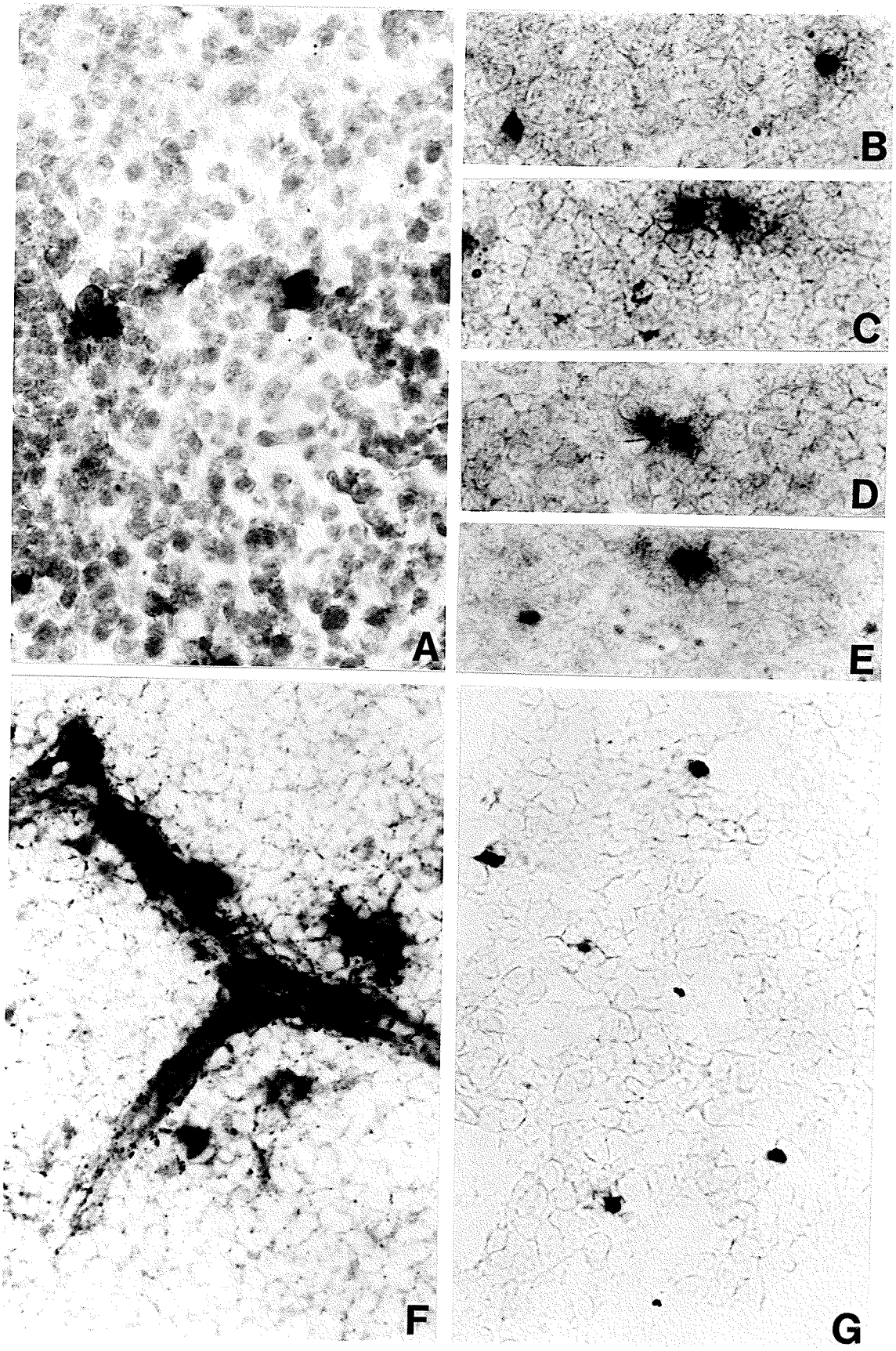
Micrographs of positively-labelled cells of the thymic medulla of a C57Bl/10ScSn mouse. The cells are typical of those found in both control and *mdx* mice (x475).

A: Three cells exhibit the dark staining initially suggestive of hybridisation of myogenin probe to myogenin mRNA using digoxigenin (DIG)-labelled probes and an AP detection system. DNase positive control (not shown) gave similar results.

B-E: Clearly positive cells were observed on all negative procedural controls, which consisted of RNase (B), no antibody to digoxigenin (C), no myogenin probe (D), and omission of both probe and DIG antibody (E), suggesting that the DIG-ISH using AP detection was not specific for myogenin mRNA.

F: Tubules in the thymus, most likely branching blood vessels, were found labelled when only the colour detection step of the procedure was performed.

G: Thymus section treated with 1M levamisole is unable to remove endogenous alkaline phosphatase activity.


A
B
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G

FIGURE 3:

Autoradiographs of C57Bl/10ScSn thymus medulla showing exposed silver grains clustered over areas containing hybridised ^{35}S-myogenin probe (hematoxylin counterstain). The pattern and distribution of grains are representative of those observed in control and *mdx* mice (A,C: x120; B,D,insets: x475).

A: Low power view of thymus section processed for *in situ* hybridisation localization of myogenin, shows collections of grains which are somewhat more dense in the medulla (between lines) than cortex.

B: High power view of an area similar to that of A. Silver grains obstruct the underlying tissue, impeding identification of cell type and number. **Inset:** Consecutive thymus section of B, with DNase treatment illustrating a similar pattern.

C: Low power view of thymus section processed for myogenin ISH after RNase treatment. There are still collections of grains present over medulla and cortex.

D: High power view of an area similar to that of C. RNase-treated thymus shows high background and silver grain aggregation (arrows) distinctly seen at lower magnifications (as in C). **Inset:** Background levels of silver grains are eliminated in the absence of probe.

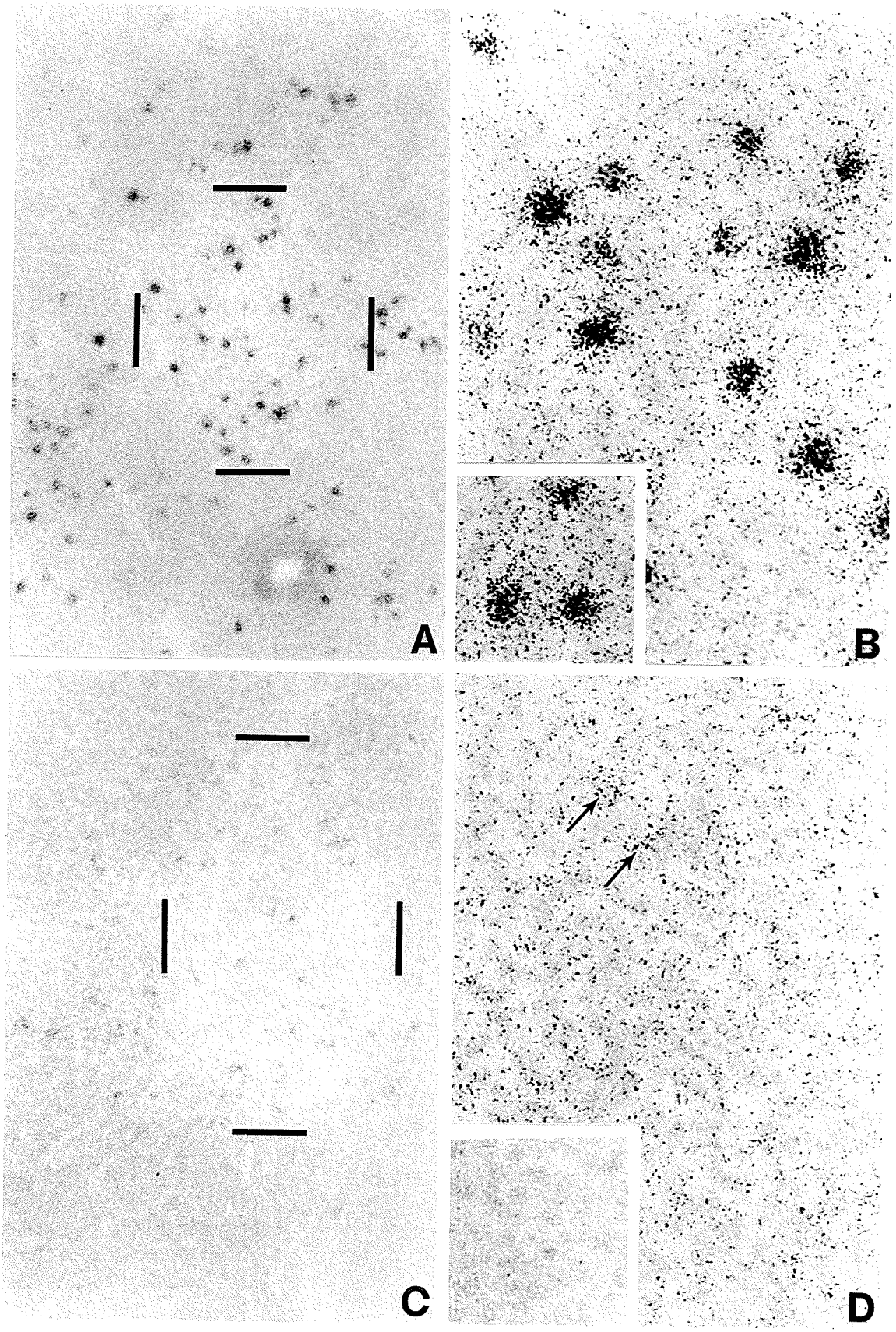
A
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FIGURE 4:

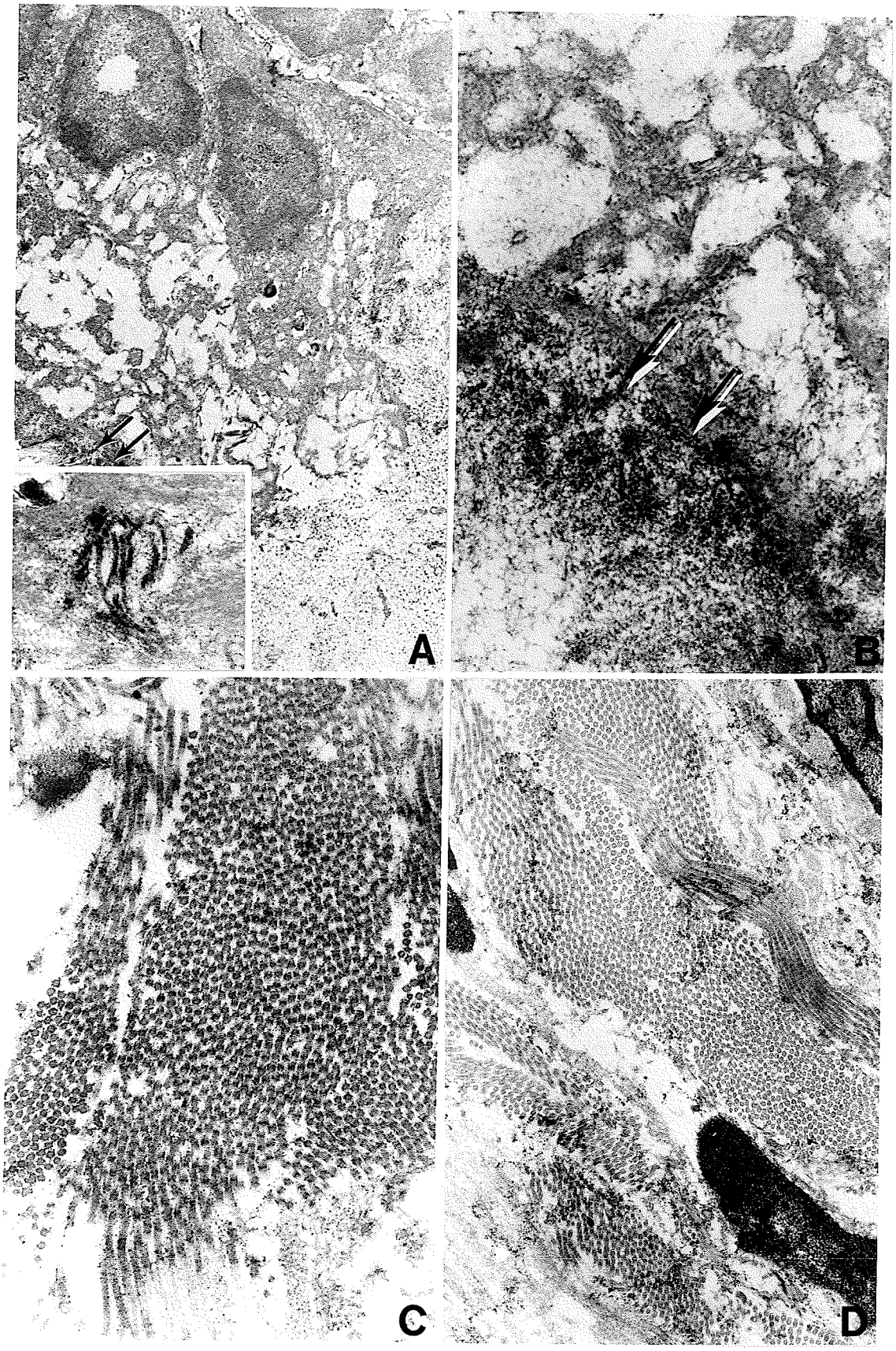
Electron micrographs of gut tissue after staining for alkaline phosphatase (AP) activity (A: x6000; inset,B,C: x24500; D: x18300).

A: Low power view of epithelial cells lining the intestinal lumen, and showing AP activity (arrows) located at the lumenal face. **Inset:** Darkly staining membranes also appeared within the basal compartment of the intestinal epithelial cells.

B: A higher power view of the same region indicated in A (arrows), illustrating electron-dense AP reaction product deposits over the glycocalyx.

C: Collagen fibers beneath intestinal epithelium also shows dense deposits from AP activity.

D: Gut tissue treated for AP activity but omitting substrate. Collagen fibers are seen at a lower magnification than in C, and the dense deposits are conspicuously absent, although some artifact (debris) is present.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D

FIGURE 5:

Electron micrographs of thymus tissue after staining for alkaline phosphatase (AP) activity (A: x2700; B,D,E: x3900; C: x18300).

A: A representative section through the thymus depicting lymphocytes, two apoptotic cells (arrows) and a blood vessel.

B: High power view of an area similar to that of A. Darkly staining periodicities (arrows) are apparent; their location is either under endothelial cells or in the basal compartment of endothelial cells around the blood vessel.

C: Periodicities (arrow) from B seen at higher magnification appear to be between two membranes but are distinct from the capillary basement membrane (arrowhead).

D: Thymus section treated for AP activity without substrate. Endothelial cells (e) line a capillary, but no periodicities are present.

E: Omission of osmium fixation causes tissue to lose much of its structural integrity. Without sufficient contrast, ultrastructural detail is not apparent and periodicities are not clearly observed. Only nuclei of the various cells show any clarity.

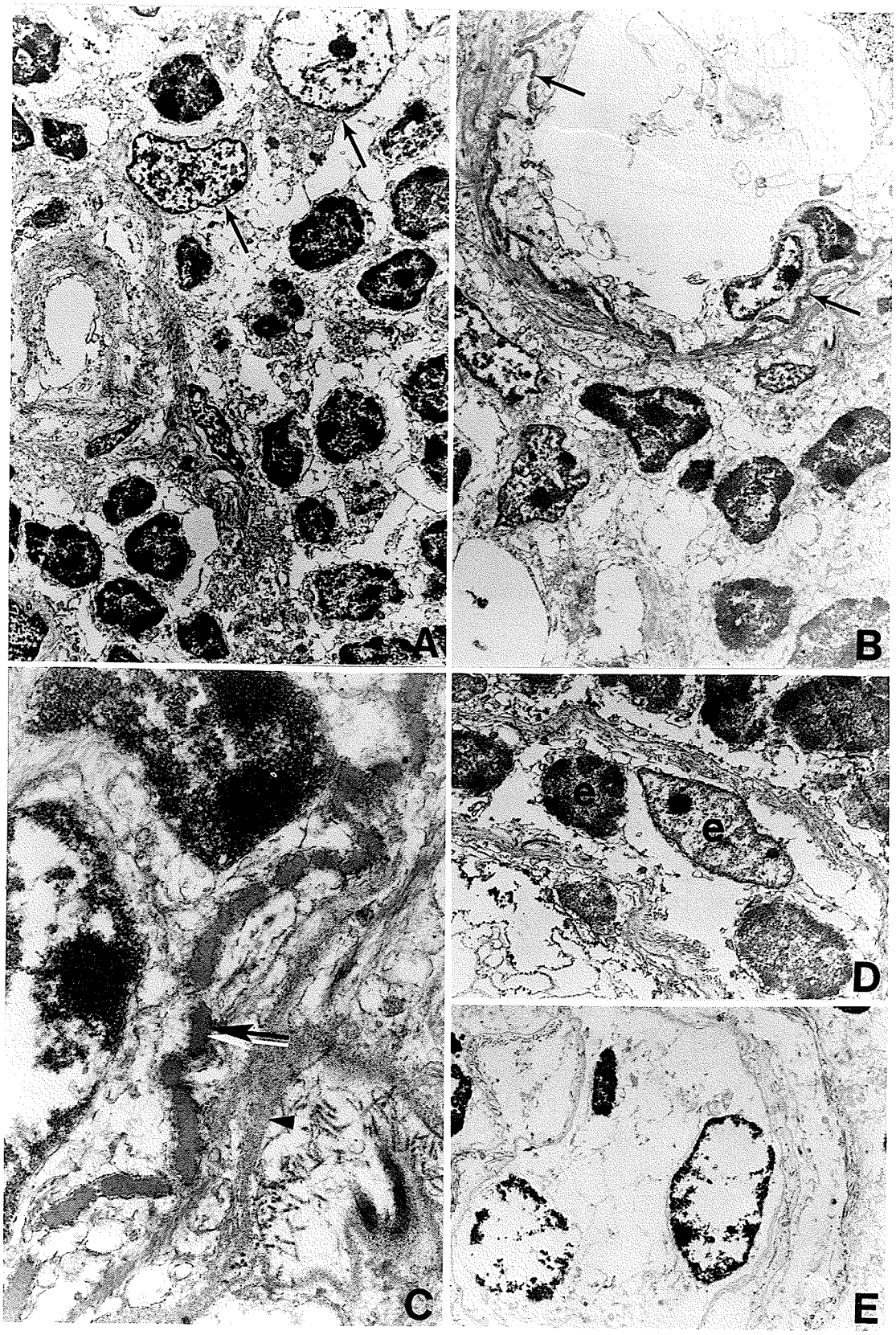
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FIGURE 6:

Electron micrographs of myoid cells in the thymus (A: x8900; B: x38500).

A: Myoid cell with myofilaments (arrows) throughout the cytoplasm. The ends of the myoid cell taper similar to the processes often observed with fluorescence microscopy.

B: Myofilaments observed in myoid cells ranged from closely packed bundles (arrow) to loosely scattered strands within the cytoplasm (as in A). Note that myoid cells contain no dense periodicities.

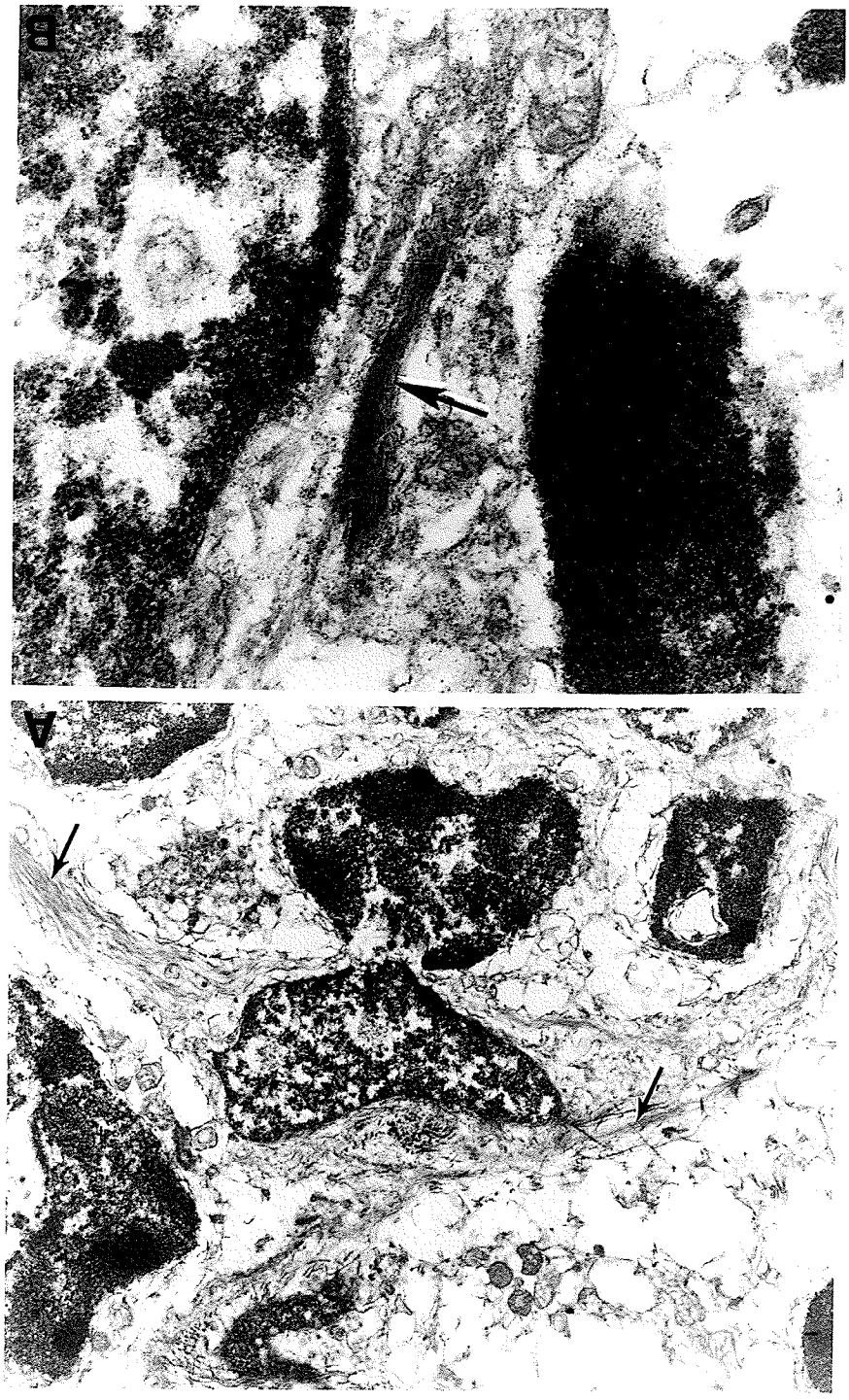

B
A

FIGURE 7:

Fluorescence micrographs of mouse TA muscle (A,B,C) and thymus (D,E,F) sections immunostained using a mouse monoclonal antibody to troponin T (TnT) (A,C: x240; B: x1190; D,E,F: x475).

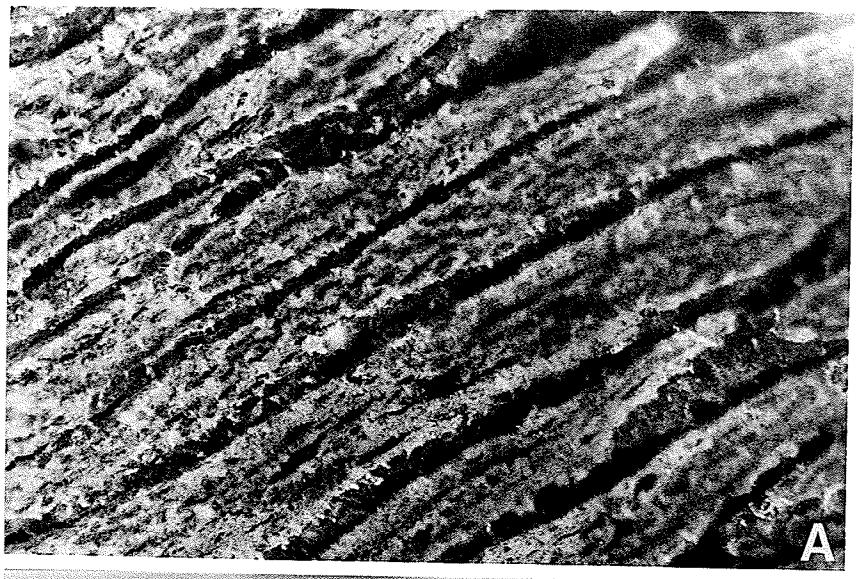
A: Staining by TnT in muscle fibers occur in a longitudinal direction.

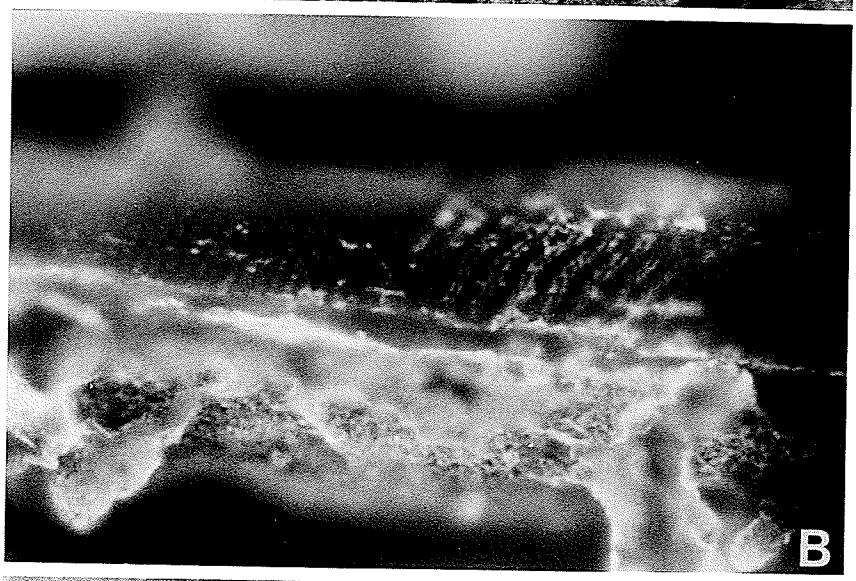
B: Specificity of TnT is demonstrated by the cross striations clearly visible in the TA muscle sections at higher magnification.

C: Muscle fibers were not stained in sections where the primary antibody (TnT) was omitted.

D & E: These 2 panels are representative of the large (D) and small (E) myoid cells observed in the 2 strains used in this study. Both types of myoid cells were generally round or oval and often had prominent cellular extensions (arrows; bar=25μm).

F: A large TnT-positive cell appears next to a blood vessel. The ring is likely to be the smooth muscle surrounding this arteriole.


A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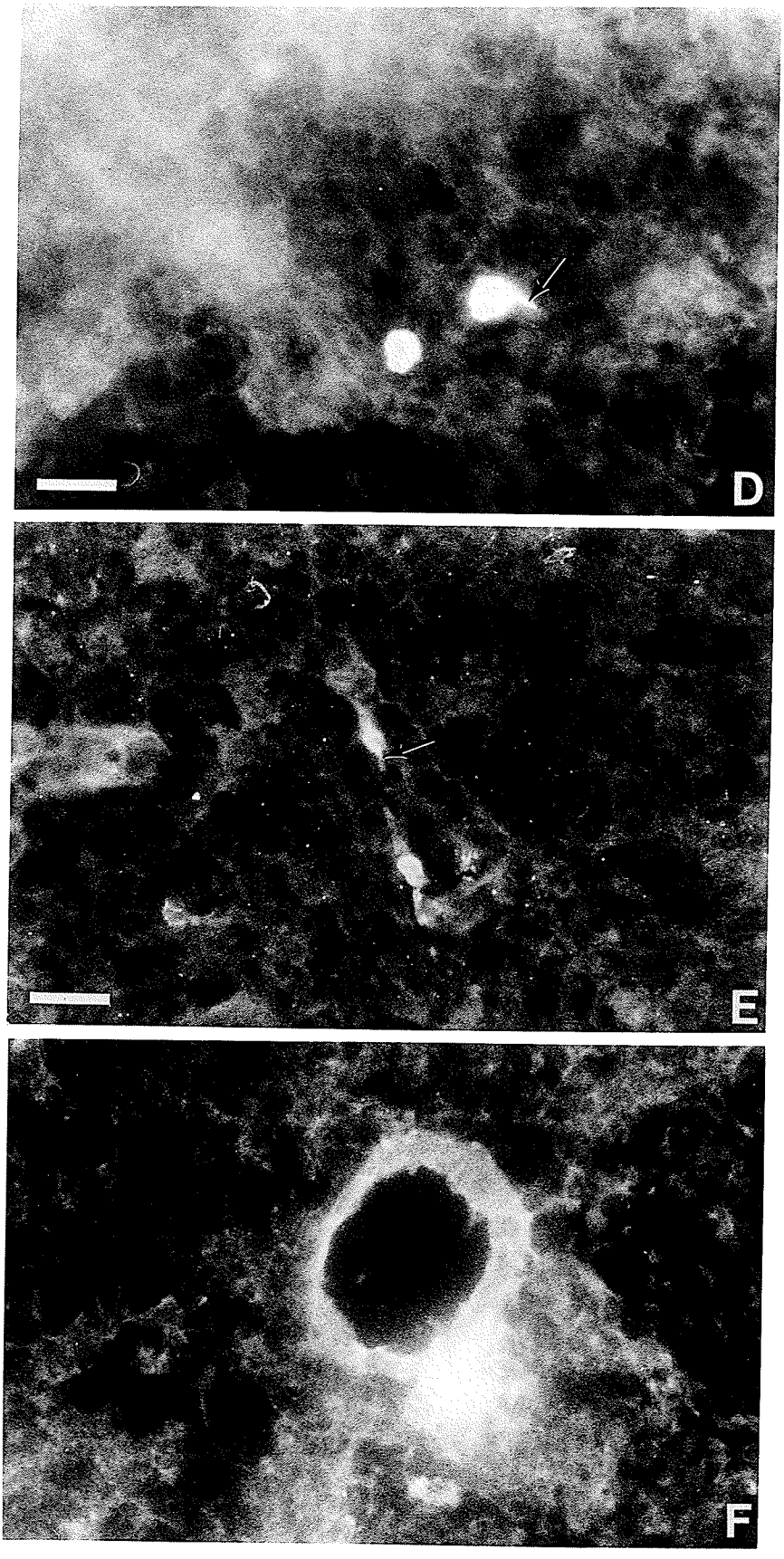
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FIGURE 8:

Graph representing the change in density of large myoid cells with respect to recovery time in Swiss Webster (SW) and *mdx* mice in response to crush injury of the right tibialis anterior muscle. (N=19 for both SW and *mdx* strains).

Initially, density dropped by day 2 of recovery then appeared to plateau 10 days after crush injury in SW mice. By contrast, large myoid cell density rose slightly 2 days post-crush and then decreased by day 10 of recovery in *mdx* mice. In both strains, the density of large cells tends to be lower (not significant) at day 10 after crush than in the original uncrushed muscle state. The densities of large myoid cells are significantly different ($p<0.0001$, 3-way ANOVA) between the 2 strains at each time point examined.

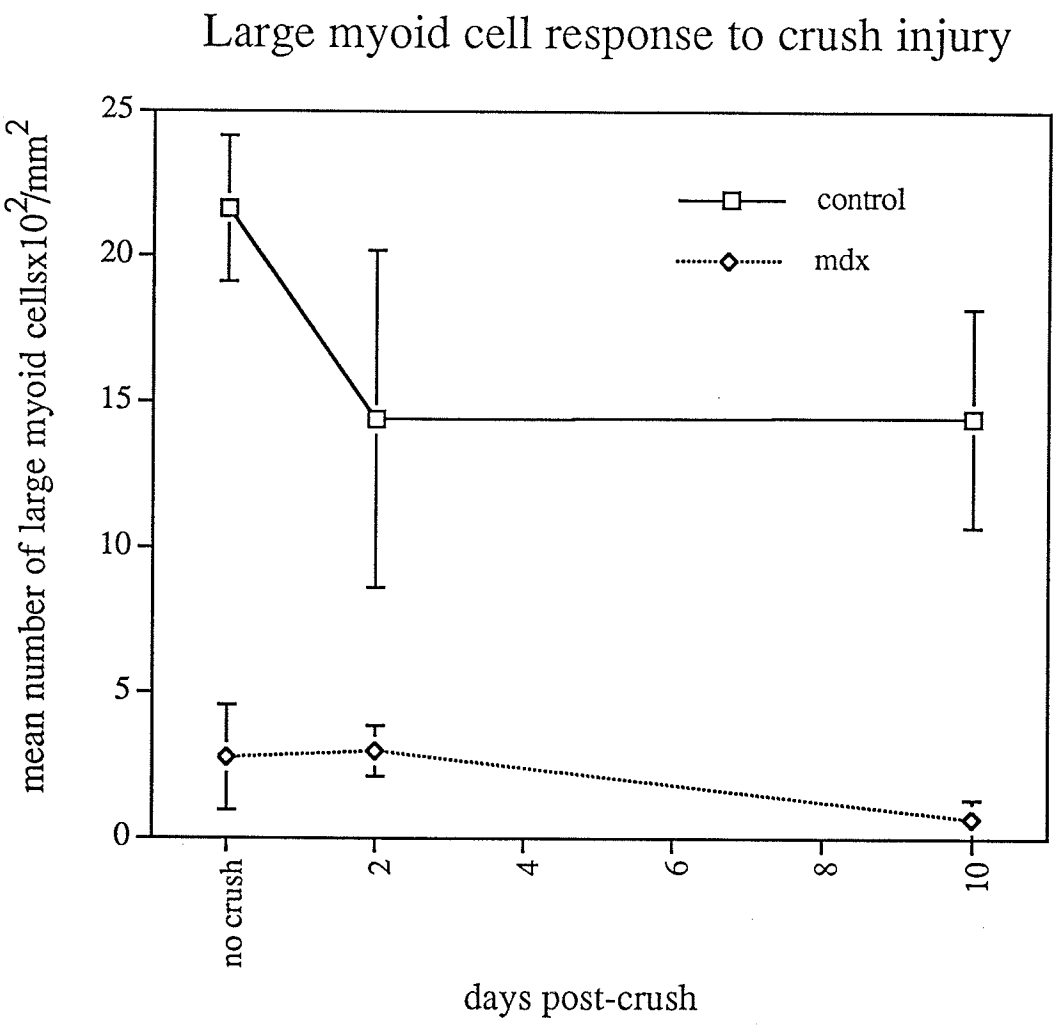
Large myoid cell response to crush injury
mean number of large myoid cellsx10^2/mm^2
25
20
15
10
5
0
control
mdx
no crush
2
4
6
8
10
days post-crush

FIGURE 9:

Muscle and thymus sections processed for autoradiography and counterstained by Gomori's trichrome method show many positive cells undergoing DNA synthesis. Silver grains obstruct the view of cells below such that positive identification of the cells is not possible by standard light microscopy staining methods (A: x240; B: x475).

A: *Mdx* TA muscle section through a region of dystrophy. Dividing mononuclear cells have infiltrated the area (arrows), but a new myotube (arrowheads), seen running parallel to some remaining intact fibers, indicates that muscle regeneration has already begun.

B: *Mdx* thymus section contains numerous cells preparing for proliferation (arrows).

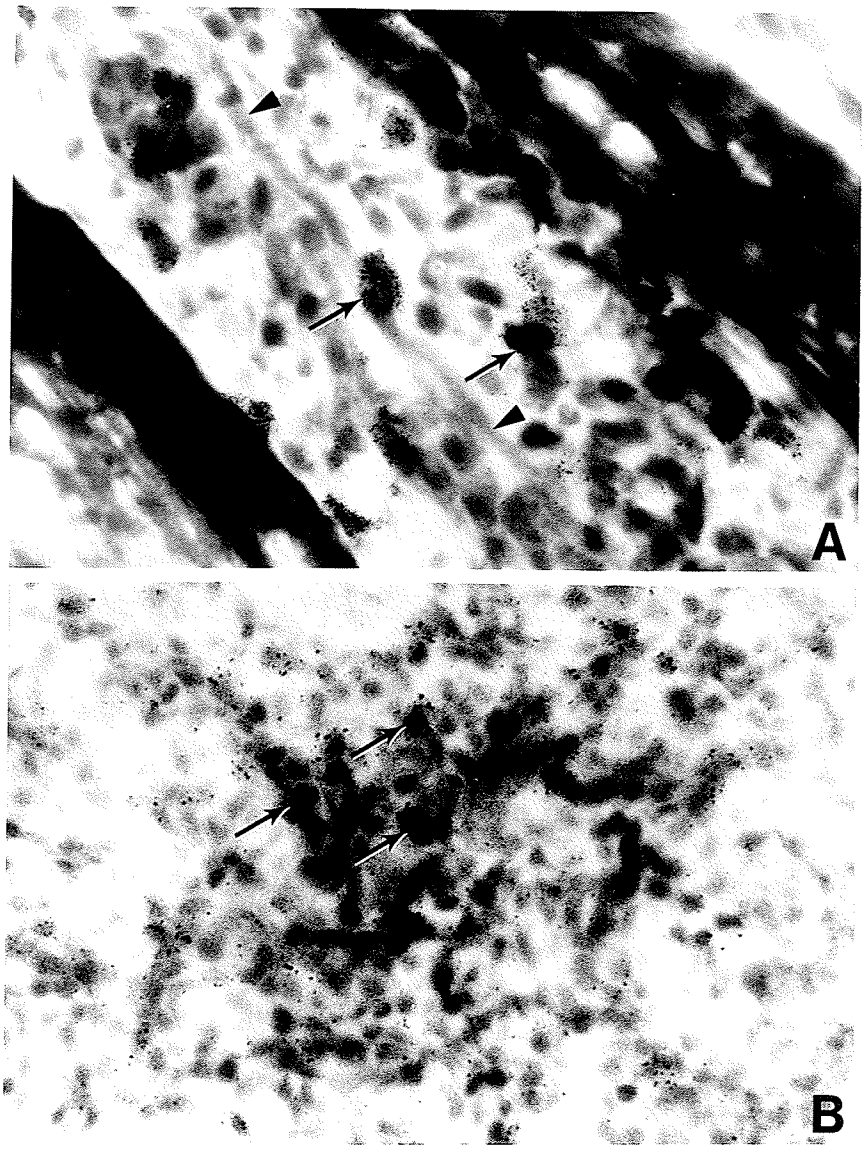
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TABLE 1: Mean ± SEM (mg) of thymus weights from control (C57Bl/10ScSn and Swiss Webster) and *mdx* mice, separated by gender. C57=C57Bl/10ScSn, SW=Swiss Webster.

strain	male	female
C57[§]	48.2 ± 6.4 (N=11)	48.6 ± 4.0 (N=15)
SW	71.1 ± 3.1 (N=11)	102.6 ± 5.5[♦] (N=8)
mdx[§]	53.2 ± 4.3[*] (N=20)	54.0 ± 3.5[*] (N=21)

[♦] indicates significance from male of same strain.
[§] indicates significance from same gender in SW.
[*] indicates significance of $p<0.05$ from SW males; all other significance was $p<0.01$.

TABLE 2: Mean ± SEM of thymus weight normalized to body weight in C57Bl/10ScSn, Swiss Webster and *mdx* mice. Gender is also provided. C57=C57Bl/10ScSn, SW=Swiss Webster; M=male, F=female.

strain	male	female
C57[§]	2.47 ± 0.53 (N=11)	2.42 ± 0.43 (N=15)
SW	3.10 ± 0.16 (N=11)	6.70 ± 0.33 (N=8)
mdx[§]	2.56 ± 0.41 (N=20)	3.50 ± 0.44 (N=21)

[§] indicates significance from SW female ($p<0.01$).

TABLE 3: Mean ± SEM ($x10^2/mm^2$) of myoid cell counts normalized to area of thymus section examined.

strain	days post-crush	large	small
normal	0 (N=6)	21.6 ± 2.5	31.6 ± 9.1
	2 (N=5)	14.4 ± 5.8	18.6 ± 4.6
	10 (N=8)	14.4 ± 3.7	23.8 ± 4.2
mdx	0 (N=4)	2.8 ± 1.8 **	7.2 ± 2.6 *
	2 (N=9)	3.0 ± 0.9 **	17.3 ± 5.5
	10 (N=6)	0.7 ± 0.7 **	15.4 ± 3.1

* indicates significance of $p\leq0.05$.
** indicates significance of $p\leq0.01$.

8. REFERENCES

Adams DH, Harvath L, Bottaro DP, Interrante R, Catalano G, Tanake Y, Strain A, Hubscher SG and S Shaw. (1994) Hepatocyte growth factor and macrophage inflammatory protein 1 beta: structurally distinct cytokines that induce rapid cytoskeletal changes and subset-preferential migration in T cells. Proceedings of the National Academy of Sciences of the USA **91(15)**: 7144-7148.

Alvarez R. (1990) Thymus of *Rana perezi*: presence of interdigitating cells. Journal of Morphology **204**: 305-312.

Anderson JE, Ovalle WK and BH Bressler. (1987) Electron microscopic and autoradiographic characterization of hindlimb muscle regeneration in the *mdx* mouse. The Anatomical Record **219**: 243-257.

Anderson JE, Liu L and E Kardami. (1991) Distinctive patterns of basic fibroblast growth factor (bFGF) distribution in degenerating and regenerating areas of dystrophic (*mdx*) striated muscles. Developmental Biology **147**: 96-109.

Aronson M. (1993) Involution of the thymus revisited: Immunological trade-offs as an adaptation to aging. Mechanisms of Ageing and Development **72**: 49-55.

Atsuta F, Kenichi S, Maruyama K and Y Shimada. (1993) Distribution of connectin (titin), nebulin and α-actinin at myotendinous junctions of chicken pectoralis muscles: An immunofluorescence and immunoelectron microscopic study. Journal of Muscle Research and Cell Motility **14**: 511-517.

Benezra R, Davis RL, Lockshon D, Turner DL and H Weintraub. (1990) The Protein Id: A negative regulator of helix-loop-helix DNA binding proteins. Cell **61**: 49-59.

Benjamini E and S Leskowitz. (1991) *Immunology: A short course.* 2nd ed. Wiley-Liss Inc: New York. pp. 1-15, 23-36.

Biben C. (1993) Myogenic factors: What might their role be? Bulletin de Institut Pasteur **91**: 161-171.

Bober E, Franz T, Arnold H-H, Peter G and P Tremblay. (1994) Pax-3 is required for the development of limb muscles: a possible role for migration of dermomyotomal muscle progenitor cells. Development **120(3)**: 603-612.

Braun T, Bober E, Winter B, Rosenthal N and HH Arnold. (1990) Myf-6, a new member of the human gene family of myogenic determination factors: Evidence for a gene cluster on chromosome 12. EMBO Journal **9**: 821-831.

Braun T, Buschhausen-Denker G, Bober E, Tannich E and HH Arnold. (1989) A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO Journal **8**: 701-709.

Braun T, Rudnicki MA, Arnold H-H and R Jaenisch. (1992) Targeted inactivation of the muscle regulatory gene myf-5 results in abnormal rib development and perinatal death. Cell **71**: 369-382.

Buckingham M. (1994) Molecular biology of muscle development. Cell **78**: 15-21.

Buckingham M, Houzelstein D, Lyons D, Ontell M, Ott M-O and D Sassoon. (1992) Expression of muscle genes in the mouse embryo. Symposia of the Society for Experimental Biology **46**: 203-217.

Bulfield G, Siller WG, Wright PAL and KJ Moore. (1984) X chromosome-linked muscular dystrophy (*mdx*) in the mouse. Proceedings of the National Academy of Sciences of the USA **81**: 1189-1192.

Campion DR. (1984) The muscle satellite cell: A review. International Review of Cytology **87**: 225-251.

Church GM, Ephrussi A, Gilbert W and S Tonegawa. (1985) Cell type specific contacts to immunoglobulin enhancers in nuclei. Nature **313**: 798-801.

Clarke AG and KA MacLennan. (1986) The many facets of thymic involution. Immunology Today **7(7-8)**: 204-205.

Cohen IR and A Cooke. (1986) Natural autoantibodies might prevent autoimmune diseases. Immunology Today **7**: 363-364.

Crouse DA, Turpen JB and JG Sharp. (1985) Thymic non-lymphoid cells. Survey of Immunologic Research **4**: 120-134.

Davis RL, Weintraub H and AB Lassar. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51**: 987-1000.

Davis RL, Cheng P-F, Lassar AB and H Weintraub. (1990) The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell **60**: 773-746.

Dias P, Dilling M and P Houghton. (1994) The molecular basis of skeletal muscle differentiation. Seminars in Diagnostic Pathology **11(1)**: 3-14.

Drenkhahn D, Unsicker K, Griesser G-H and U Schumacher. (1978) Different myosins in myoid and entodermal reticular epithelial cells of the thymus. Cell and Tissue Research **187**: 97-103.

Drenkhahn D, von Gaudecker B, Muller-Hermelink HK, Unsicker K and U Groschel-Stewart. (1979) Myosin and actin containing cells in the human postnatal thymus. Virchows Archiv B [Cell Pathology] **32**: 33-45.

Edmonson DG and EN Olson. (1989) A gene with homology to the *myc* similarity region of MyoD1 is expressed during myogenesis and is suffcient to activate the muscle differentiation program. Genes and Development **3**: 628-640.

Eftimie R, Brenner HR and A Buonanno. (1991) Myogenin and MyoD join a family of skeletal muscle genes regulated by electrical activity. Proceedings of the National Academy of Sciences of the USA **88(4)**: 1349-1353.

Florini JR, Ewton DZ and KA Magri. (1991) Hormones, growth factors and myogenic differentiation. Annual Review of Physiology **53**: 201-216.

Franz T, Kothary R, MAH Surani, Halata Z and M Grim. (1993) The splotch mutation interferes with muscle development in the limbs. Anatomy and Embryology **187(3)**: 153-160.

Garrett KL and JE Anderson. (1995) Co-localisation of bFGF and the myogenic regulatory gene myogenin in dystrophic *mdx* muscle precursors and young myotubes *in vivo*. Developmental Biology (in press)

Guérette B, Asselin I, Vilquin JT, Roy R and JP Tremblay. (1995) Lymphocyte infiltration following allo- and xenomyoblast transplantation in *mdx* mice. Muscle and Nerve **18**: 39-51.

Gilbert SF. (1991) *Developmental Biology*. Sinauer Associates, Inc: Sunderland. pp.201-209.

Gospodarowicz D, Ferrara N, Schweigerer L and G Neufeld. (1987) Structural characterization and biological functions of fibroblast growth factor. Endocrinology Review **8**: 95-114.

Gomori G. (1939) Microtechnical demonstration of phosphatase in tissue sections. Proceedings of the Society of Experimental Medicine **42**: 23-26.

Grounds MD, Garrett KL and MW Beilharz. (1992a) The transcription of MyoD1 and myogenin in thymic cells *in vivo*. Experimental Cell Research **198**: 357-361.

Grounds MD, Garrett KL, Lai MC, Wright WE and MW Beilharz. (1992b) Identification of skeletal muscle precursor cells *in vivo* by use of MyoD1 and myogenin probes. Cell Tissue Research **267**: 99-104.

Hannon K, Smith CK, Bales KR and RF Santerre. (1992) Temporal and quantitative analysis of myogenic regulatory and growth factor gene expression in the developing mouse embryo. Developmental Biology **151**: 137-144.

Hammar JA. (1905) Der gegenwärtige stand der morphologie und physiologie der thymusdrüse. Wien Med Wschr **2**: 2910-2916.

Hasty P, Bradley A, Morris JH, Edmondson DG, Venuti JM, Olson EN and WH Klein. (1993) Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature **364**: 501-506.

Henderson C. (1989) Aminoalkylsilane: An inexpensive, simple preparation for slide adhesion. Journal of Histotechnology **12(2)**: 123-124.

Henry K. (1966) Mucin secretion of striated muscle in the human thymus. Lancet **1**: 183-185.

Hirokawa K, Utsuyama M, Kasai M, Konno A, Kurashima C and E Moriizumi. (1990) Age-related hyperplasia of the thymus and T-cell system in the Buffalo rat. Virchows Archiv B [Cell Pathology] **59**: 38-47.

Hughes SM, Taylor JM, Tapscott SJ, Gurley C M, Carter WJ and CA Peterson. (1993) Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult muscle controlled by innervation and hormones. Development **118**: 1137-1147.

Hugon J and M Borgers (1966). A direct lead method for the electron microscopic visualization of alkaline phosphatase activity. Journal of Histochemistry and Cytochemistry **14(5)**: 429-431.

Itoh T. (1983) Establishment of a myoid cell clone from rat thymus. Cell and Tissue Research **231**: 39-47.

Johnson SE and RE Allen. (1993) Proliferating cell nuclear antigen (PCNA) is expressed in activated rat skeletal muscle satellite cells. Journal of Cellular Physiology **154**: 39-43.

Junqueira LC, Carneiro J and RO Kelley. (1992) *Basic Histology.* 7th ed. Appleton & Lange: Conneticut. pp.261-269.

Kaiserling E, Stein H and HK Müller-Hermelink. (1974) Interdigitating reticulum cells in the human thymus. Cell Tissue Research **155**: 47-55.

Kamo I, Tada-Kikuchi A, Furukawa S, Iwasaki Y, Nonaka I, Satoyoshi E and T Itoh. (1985) Effects of thymic myoid cell culture supernatant on cells from lymphatic tissues. Cellular Immunology **94**: 587-597.

Kirchner T, Tzartos S, Hoppe F, Schalke B, Wekerle H and HK Müller-Hermelink. (1988) Acetylcholine receptor-related antigenic determinants in tumor-free thymuses and thymic epithelial tumors. American Journal of Pathology **130(2)**: 268-280.

Knudsen K, McElwee SA and L Myers. (1990) A role for the neural cell adhesion molecule, NCAM, in myoblast interaction during myogenesis. Developmental Biology **138**: 159-168.

Lacey AJ. (1989) *Light Microscopy in Biology: A Practical Approach.* Oxford University Press: Oxford. pp.103-136.

Latker CH, Shinowara NL, Miller JC and SI Rapoport. (1987) Differential localization of alkaline phosphatase in barrier tissues of the frog and rat nervous systems: A cytochemical and biochemical study. Journal of Comparative Neurology **264**: 291-302.

Lin-Jones JH, Stern HM and SD Hauschka. (1994) An RT-PCR analysis of myogenic determination factor expression during chick somite and limb bud development. Journal of Cellular Biochemistry Supplement 18D, W235 (ABST): 501.

Mauro A. (1961) Satellite cells of skeletal muscle fibres. Journal of Biophysical and Biochemical Cytology **9**: 493-495.

Mayer S. (1888) Zur lehre von der schildrüse und thymus bei den amphibien. Anat Anz **3**: 97-103.

McGeachie JK and MD Grounds. (1987) Initiation and duration of muscle precursor replication after mild and severe injury to skeletal muscle of mice. Cell and Tissue Research **248**: 125-130.

McGeachie JK, Grounds MD, Partridge TA and JE Morgan. (1993) Age-related changes in replication of myogenic cells in *mdx* mice: quantitative autoradiographic studies. Journal of the Neurological Sciences **119**: 169-179.

McIntosh LM and JE Anderson. (1995) Hypothyroidism prolongs and increases *mdx* muscle precursor proliferation and delays myotube formation in normal and dystrophic limb muscle. Cell Biochemistry **73** (in press).

McIntosh LM, Pernitsky AN and JE Anderson. (1994) The effects of altered metabolism (hypothyroidism) on muscle repair in the *mdx* dystrophic mouse. Muscle and Nerve **17**: 444-453.

Mege RM, Goudou D, Diaz C, Nicolet M, Garcia L, Geraud G and F Rieger. (1992) N-cadherin and N-CAM in myoblast fusion: compared localisation and effect of blockade by peptides and antibodies. Journal of Cell Science **103**: 897-906.

Melms A, Schalke BCG, Kirchner T, Müller-Hermelink HK, Albert E and H Wekerle. (1988) Thymus in myasthenia gravis. Isolation of T-lymphocyte lines specific for the nicotinic acetylcholine receptor from thymuses of myasthenic patients. Journal of Clinical Investigation **81**: 902-908.

Meinl E, Klinkert WEF and H Wekerle. (1991) The thymus in myasthenia gravis: Changes typical for the human disease are absent in experimental autoimmune myasthenia gravis of the Lewis rat. American Journal of Pathology **139(5)**: 995-1008.

Miller JB, Everitt EA, Smith TH, Block NE and JA Dominov. (1993) Cellular and molecular diversity in skeletal muscle development: News from *in vitro* and *in vivo*. BioEssays **15(3)**: 191-196.

Miner JH and B Wold. (1990) Herculin, a fourth member of the MyoD family of myogenic regulatory genes. Proceedings of the National Academy of Sciences of the USA **87**: 1089-1093.

Mitchell CA, McGeachie JK and MD Grounds. (1992) Cellular differences in regeneration of murine skeletal muscle: a quantitative histological study in SJL/J and BALB/c mice. Cell Tissue Research **269**: 159-166.

Moore KL. (1989) *Before We Are Born: Basic Embryology and Birth Defects*. 3rd ed. WB Saunders Company: Philadelphia. pp.50, 240-243.

Müller-Hermelink HK and A Marx. (1994) The relationship of the thymus to the pathologies of myasthenia gravis. Muscle and Nerve Supplement 1 (ABST): S64.

Murphy LJ, Bell GI, Duckworth ML and HG Friesen. (1987) Identification, characterization and regulation of a rat complementary deoxyribonucleic acid which encodes insulin-like growth factor-I. Endocrinology **121(2)**: 684-691.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, Weintraub H and D Baltimore. (1989) Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58**: 537-544.

Murre C, Voronova A and D Baltimore. (1991) B-cell- and myocyte-specific E2-box-binding factors contain E12/E47-like subunits. Molecular and Cellular Biology **11**: 1156-1160.

Nabeshima Y, Hanaoka K, Hayasaka M, Esumi E, Li S, Nonaka I and Y Nabeshima. (1993) Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature **364**: 532-535.

Olson EN. (1990) MyoD family: A paradigm for development? Genes and Development **4**: 1454-1461.

Olson E. (1992a) Interplay between proliferation and differentiation within the myogenic lineage. Developmental Biology **154**: 261-272.

Olson E. (1992b) Activation of muscle-specific transcription by myogenic helix-loop-helix proteins. Symposia of the Society for Experimental Biology **46**: 331-341.

Olson EN. (1993) Regulation of muscle transcription by the MyoD family: The heart of the matter. Circulation Research **72**: 1-6.

Olson EN, Brennan TJ, Chakraborty T, Cheng T-C, Cserjesi P, Edmondson D, James G and L Li. (1991) Molecular control of myogenesis: Antagonism between growth and differentiation. Molecular and Cellular Biochemistry **104**: 7-13.

Ontell M, Ontell MP, Sopper MM, Mallonga R, Lyons G and M Buckingham. (1993) Contractile protein gene expression in primary myotubes of embryonic mouse hindlimb muscles. Development **117**: 1435-1444.

Pappenheimer AM. (1913) Further studies in the histology of the thymus. American Journal of Anatomy **14**: 299-332.

Paulsen DF. (1993) *Basic Histology*. 2nd ed. Appleton & Lange: Conneticut. pp.181-188.

Perico N, Rossini M, Imberti O and G Remuzzi. (1991) Thymus-mediated immune tolerance to renal allograft is donor but not tissue specific. Journal of the American Society of Nephrology **2**: 1063-1071.

Pinney DF, Pearson-White SH, Konieczny SF, Latham KE and CP Emerson. (1988) Myogenic lineage determination and differentiation: Evidence for a regulatory gene pathway. Cell **53**: 781-793.

Polak JM and SV Noorden. (1987) *An introduction to immunocytochemistry: current techniques and problems*. Revised ed. Oxford University Press: Oxford.

Puchtler H, Meloan SN, Branch BW and S Gropp (1975). Myoepithelial Cells in Human Thymus: Staining, Polarization and Fluorescence Microscopic Studies. Histochemistry **45**: 163-176.

Raviola E and G Raviola. (1967) Striated muscle cells in the thymus of reptiles and birds: An electron microscopic study. American Journal of Anatomy **121**: 623-646.

Rhodes JH and SF Konieczny. (1989) Identification of MRF4: A new member of the muscle regulatory factor gene family. Genes and Development **3**: 2050-2061.

Rudnicki MA, Braun T, Hinuma S and R Jaenisch. (1992) Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene myf-5 and results in apparently normal muscle development. Cell **71**: 383-390.

Rudnicki MA, Schnegelsberg PNJ, Stead RH, Braun T, Arnold H-H and R Jaenisch. (1993) MyoD or myf-5 is required for the formation of skeletal muscle. Cell **75**: 1351-1359.

Sambrook J, Fritsch EF and T Maniatis. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press: New York.

Sassoon D, Lyons G, Wright WE, Lin V, Lassar A, Weintraub H and M Buckingham. (1989) Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. Nature **341**: 303-307.

Sato T and N Tamaoki. (1989) Myoid cells in the human thymus and thymoma revealed by three different immunohistochemical markers for striated muscle. Acta Pathologica Japonica **39(8)**: 509-519.

Schluep M, Wilcox N, Vincent A, Dhoot GK and J Newsom-Davis. (1987) Acetylcholine receptors in human thymic myoid cells in situ: An immunohistological study. Annals of Neurology **22**: 212-222.

Schönbeck S, Padberg F, Hohlfeld R and H Wekerle. (1992) Transplantation of thymic autoimmune microenvironment to severe combined immunodeficiency mice. Journal of Clinical Investigation **90**: 245-250.

Schuurman H-J, Nagelkerken L, De Weger RA and J Rozing. (1991) Age-related involution: Significance of a physiologic process. Thymus **18**: 1-6.

Seifert R and B Christ. (1990) On the differentiation and origin of myoid cells in the avian thymus. Anatomy and Embryology **181**: 287-298.

Smith TH, Blick NE, Rhodes SJ, Konieczny SF and JB Miller. (1993) A unique pattern of expression of the four muscle regulatory factor proteins distinguishes somitic from embryonic, fetal and newforn mouse myogenic cells. Development **177**: 1125-1133.

Smith CK, Janney MJ and RE Allen. (1994) Temporal expression of myogenic regulatory genes during activation, proliferation and differentiation of rat skeletal muscle satellite cells. Journal of Cellular Physiology **159**: 379-385.

Stockdale FE. (1992) Myogenic cell lineages. Developmental Biology **154**: 284-298.

Stockdale FE, Miller JB, Feldman JL, Lamson G and J Hager. (1989) Myogenic cell lineages: Commitment and modulation during differentiation of avian muscle. In: *Cellular and Molecular Biology of Muscle Development*. Alan R. Liss, Inc. pp.3-13.

Stockdale FE. (1990) Myoblast diversity and the formation of the early limb musculature. Annals of the New York Academy of Sciences **599**: 111-118.

Tajbakhsh S and ME Buckingham. (1994) Mouse limb muscle is determined in the absence of the earliest myogenic factor myf-5. Proceedings of the National Academy of Sciences of the USA **91**: 747-751.

Tapscott SJ, Lassar AB, Davis RL and H Weintraub. (1989) 5-bromo-2'-deoxyuridine blocks myogenesis by extinguishing expression of MyoD1. Science **245(4917)**: 532-536.

Tapscott SJ and H Weintraub. (1991) MyoD and the regulation of myogenesis by helix-loop-helix proteins. Journal of Clinical Investigation **87**: 1133-1138.

Töró I, Oläh I, Röhlich P and SZ Virágh. (1969) Electron microscopic observations on myoid cells of the frog's thymus. Anatomical Record **165**: 329-342.

Vaidya TB, Phodes SJ, Taparowsky EJ and SF Konieczny. (1989) Fibroblast growth factor and transforming growth factor ß repress transcription of the myogenic regulatory gene MyoD1. Molecular and Cellular Biology **9**: 3576-3579.

Valikova TA, Gnezditskaya EV, Beletskaya LV, Shiyanevsii AY, Pekarskii VV and GT Dambaev. (1982) Myoid cells of the thymus in patients with myopathy. Bulletin of Experimental Biology and Medicine **93**: 104-106.

Van de Velde RL and NB Friedman. (1970) Thymic myoid cells and myasthenia gravis. American Journal of Pathology **59(2)**: 347-367.

Vincent A, Scadding GK, Thomas HC and J Newsom-Davis. (1978) *In vitro* synthesis of anti-acetylcholine-receptor antibody by thymic lymphocytes in myasthenia gravis. Lancet **i**: 305-307.

Wang Y, Benezra R and DA Sassoon. (1992) Id expression during mouse development: A role in morphogenesis. Developmental Dynamics **194**: 222-230.

Webster C and HM Blau. (1990) Accelerated age-related decline in replicative life-span of Duchenne muscular dystrophy myoblasts: implications for cell and gene therapy. Somatic Cell Molecular Genetics **16**: 557-565.

Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell, TK, Turner D, Rupp R, Hollenberg S, Zhuang Y and A Lassar. (1991) The MyoD gene family: Nodal point during specification of the muscle cell lineage. Science **251**: 761-766.

Wekerle H, Hohlfeld R, Ketelsen U-P, Kalden JR and I Kalies. (1981) Thymic myogenesis, T-lymphocytes and the pathogenesis of myasthenia gravis. Annals of the New York Academy of Sciences **337**: 455-476.

Wekerle H, Ketelsen U-P, Zurn AD and EW Fulpius. (1978) Intrathymic pathogenesis of myasthenia gravis: Transient expression of acetylcholine receptors on thymus-derived myogenic cells. European Journal of Immunology **8**: 579-582.

Wekerle H, Paterson B, Ketelsen U-P and M Feldman. (1975) Striated muscle fibers differentiate in monolayer cultures of adult thymus reticulum. Nature **256**: 493.

Wilkinson DG. (1992) *In Situ Hybridization: A Practical Approach.* Oxford University Press: Oxford. pp. 1-32.

Williams PL, Warwick R, Dyson M and LH Bannister. (1989) *Gray's Anatomy.* 37th ed. Churchill Livingstone, Inc: Edinburgh. pp. 554-557.

Wright WE, Sassoon DA and VK Lin. (1989) Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell **56**: 607-617.

Zacharias JM and JE Anderson. (1991) Muscle regeneration after imposed injury is better in younger than older *mdx* dystrophic mice. Journal of the Neurological Sciences **104**: 190-196.

APPENDIX A: SILANATION OF SLIDES

Glass slides were washed thoroughly with detergent and baked at >180oC over night. In a fumehood, a 2% solution of aminoalkylsilane (AAS) in dry acetone was prepared immediately prior to use, with 3-aminopropyltriethoxysilane (Sigma). Slides were dipped into the 2% AAS solution for 10 seconds then taken through 3 washes of DEPC-treated water (10 seconds or more in each) and air dried. Slides were stored in slide boxes at room temperature until required. The 2% AAS solution is flammable and corrosive and should only be used once (good for 400 slides); used AAS solution should be stored in a brown bottle until enough is collected for disposal through the safety office.

APPENDIX B: BUFFERS AND REAGENTS

DIGOXIGENIN BUFFERS

Buffer 1

	to make 2500mL	to make 4L
100mM Maleic acid	29.018g	46.428g
150mM sodium chloride	21.915g	35.064g
NaOH	~20g to pH 7.5	~30g to pH 7.5

Buffer 2

	to make 100mL
1% blocking reagent in buffer 1	1g

heat at 50-70°C to help blocking reagent dissolve
store aliquots frozen at -20°C

10% stock - 10g in 100mL buffer 1
store at -20°C

Buffer 3

	to make 1000mL
100mM Tris	12.11g Tris base
100mM sodium chloride	33.3mL 3M or 20mL 5M or 5.844g
50mM magnesium chloride	50mL 1M or 10.165g

pH 9.5
Use Tris base to reduce the formation of a precipitate that is produced using NaOH to increase the pH of the solution made from Tris at a lower pH.
If precipitate forms, filter the solution.

Buffer 4

	to make 1000mL
10mM Tris	10mL 1M
1mM EDTA	2mL 0.5M

pH 8.0

BUFFERS

1M Tris

121.1g Tris base in 800mL ddH_2O
pH w/ conc. hydrochloric acid (approx.)
pH 7.4 70mL
pH 7.6 60mL
pH 8.0 42mL

0.5M ethylenediaminetetraacetic acid (EDTA)

186.1g in 800mL ddH_2O
pH to 8.0 w/ 10M sodium hydroxide or approx. 10g of NaOH pellets, autoclave.

1M calcium chloride ($CaCl_2$)

25.94g calcium chloride in 200mL
sterilize through a 0.22m millipore filter
store aliquots at -20°C

1M magnesium chloride ($MgCl_2$)

Dissolve 203.3g of $MgCl_2.6H_2O$ in 1L of ddH_2O, autoclave.

5M sodium chloride (NaCl)

146.25g NaCl in 500mL ddH_2O

3M sodium acetate (NaAc)

40.81g sodium acetate.$3H_2O$ in 80mL ddH_2O
pH to 5.2 w/ glacial acetic acid
Make up to 100mL and autoclave

10% sodium dodecyl sulphate (SDS)

50g SDS in 500mL ddH_2O
pH to 7.0 w/ a few drops of conc hydrochloric acid

Tris ethylenediaminetetraacetic acid (TE)

working conc	10x stock - to make 1000mL
10mM Tris	12.11g
1mM EDTA	3.72g

adjust pH 8.0 w/ HCl and autoclave

20x standard saline citrate (SSC)

	to make 1000mL
3M NaCl	175.3g
0.3M trisodium citrate	88.2g

make up to 800mL w/ ddH_2O
pH 7 w/ 1M HCl
adjust volume to 1000mL and autoclave

Phosphate buffered saline (PBS)

working conc (pH 7)	10x stock - to make 1000mL
137mM NaCl	80g
2.7mM KCl	2g
10mM $Na_2HPO_4.7H_2O$	27.2g
1.76mM KH_2PO_4	2.4g

make to 1000mL
adjust to pH 7.4 w/ NaOH
NB: at 4°C 10xPBS precipitates to crystals

APPENDIX C: THYMUS WEIGHT RESPONSE TO MUSCLE CRUSH

Mean ± SEM of thymus weight normalized to body weight in untreated and TA-crushed control (C57Bl/10ScSn and Swiss Webster) and *mdx* mice. C57=C57Bl/10ScSn, SW=Swiss Webster.

strain	no crush	2 days post-crush[§]
C57[♦]	4.91 ± 0.21 (N=3)	4.46 ± 0.14 (N=6)
SW	3.36 ± 0.17 (N=6)	2.80 ± 0.24 (N=5)
mdx[♦]	6.25 ± 0.12 (N=4)	4.61 ± 0.16 (N=9)

[§] indicates significance from no crush condition ($p<0.0001$) in same mouse strain.
[♦] indicates significance from same crush status in SW ($p<0.0001$).