AN EM INVESTIGATION OF SKELETAL MUSCLE REGENERATION IN CULTURED EXPLANTS

by

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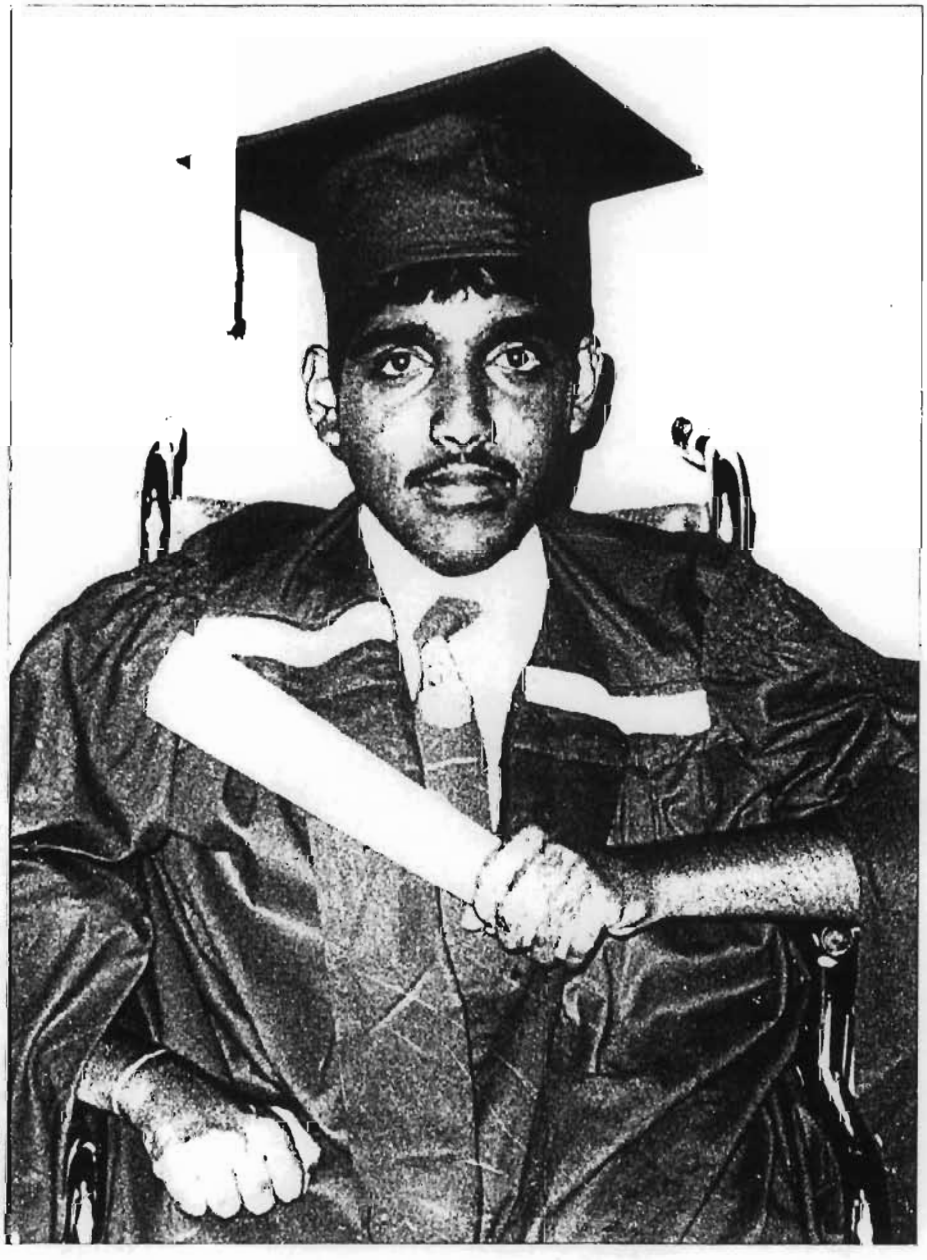
DEDICATION

This thesis is dedicated to my late son, Suriapregasen Naidoo who was afflicted by Duchenne muscular dystrophy, and to all the physically disabled persons tormented by genetic disorders and to their parents and loved ones who share the trials of such misfortune.



SURIAPREGASEN NAIDOO: Born April, 1969 and lost in January, 1988

"...there was a purpose to his life."

PREFACE

These studies represent original work by the author and have not been submitted in any form to another University. The work from other researchers, wherever used has been duly acknowledged in the text.

The research described in this thesis was conducted independently as the original supervisor, Professor A. Moosa, the former head of the Department of Paediatrics, University of Natal, left the institution to take up a post in Kuwait. The research work was carried out in the Department of Human Physiology and Physiological Chemistry, University of Durban-Westville and at the EM Units based in the Department of Physiology, Medical School, University of Natal and at University of Durban-Westville. Professor P. Gathiram who joined the department last year, having come from the Natal Medical School, was appointed in 1994 as a supervisor who undertook to oversee the completion of the thesis. Professor Gathiram could also bear testimony that the research was conducted independently by the author.

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ABSTRACT

Research involving myoblast transplant and gene transfer therapy, both as possible answers for muscular dystrophy, depend on a sound knowledge of the mechanism of muscle regeneration. Therefore, the current study was undertaken to dispel the confusion that exists in the understanding of the origin of myoblasts during muscle regeneration.

An EM investigation of muscle regeneration in cultured muscle explants made possible for the first time, a record of the early events occurring during regeneration. The regeneration processes, occurring within cultured hamster and human muscle explants for 10 consecutive days of incubation, were studied under the EM. Pre-incubation, uncultured specimens served as controls.

The major revelations were that euchromatic myonuclei undergoing dense granulation and heterochromacity, denoting nuclear activation after incubation, herald the transformation of these myonuclei to the precursor cells of muscle regeneration or future myoblasts. Partial transformational forms of myonuclei towards a presumptive myoblast were clearly observed in the study. This is how the classical satellite cells are formed in adult muscle, with the cytoplasm around the young cells being apparently new cytoplasm formed by the nucleus. During regeneration, the myoblasts increased in number, underwent fusion and formed multinucleate myotubes. Myoblasts and myotubes also exhibited phagocytic behaviour during the course of development to a young myofibre. It is not certain, at this stage, how the myoblasts proliferated as mitotic figures were not observed. However, morphological evidence suggesting amitotic division of myonuclei by segmentation, perhaps explains the possible proliferation of the myoblasts from new myonuclei thus formed. Myotubes may also be formed

from mononucleated myoblasts by apparent amitotic division of their nuclei, and this is, possibly, the answer to the intriguing question: of how myonucleation occur within myotubes?

When the myoblast resources from myonuclear derivation are depleted, the muscle would appear to have the potential to develop "new generation cytoplasm" and "new generation nuclei" in apparent association with sarcoplasmic organelles, mainly mitochondria by yet unknown mechanisms. The new nuclei and cytoplasm, thus formed, lead to the formation of "new generation cells". Clusters or chains of irregular and bizarre nuclei within myotubes were apparently formed by fusion of mononucleated newly generated cells or from newly generated multinuclei within newly generated cytoplasm. This is, perhaps, the way in which bizarre nuclei were derived in pathological muscle. Possible amitotic division was also apparent in the these irregular nuclei. The mitochondria, apart from being the power house of the cells in biochemical terms, seemed in some way associated with the development of structural elements of the muscle cells such as new plasma, new nuclei and new fibrous material. These assumptions need biochemical or immunocytochemical validation!

Finally, it is hoped that information gained in this study, will help towards a better interpretation of the morphological changes observed in the muscle of experimental subjects undergoing therapeutic trials and in pathological muscle from patients. Furthermore, if the results of this study are confirmed in the future at other research stations, the researchers involved in the search for cure will have to re-orientate their therapeutic design and targets, focussing attention on the myonuclei and not the satellite cells.

Keywords: regeneration; skeletal muscle; myoblasts; satellite cell; mitochondria; phagocytic myoblasts.

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CHAPTER 1

INTRODUCTION

1.1 Background

Neuromuscular diseases, especially muscular dystrophy, provided the research field for this study on muscle regeneration. Muscular dystrophy, an insidious muscle wasting disease due to a genetic disorder, up to the present day, continues to defy modern medical science. Nevertheless, dedicated efforts of research scientists from around the world are producing exciting and illuminating discoveries, bringing hope and encouragement for the muscular dystrophy patients and their families.

Muscular dystrophy research has been in progress for many decades (Dubowitz, 1989a). Most research has been concentrated on Duchenne muscular dystrophy (DMD), this being the most common and debilitating of the dystrophies. It is anticipated that if science succeeds in unravelling the mysteries of DMD, then the understanding and solutions for most other genetic diseases would inevitably ensue (Kunkel, 1989).

Research, only recently, established that Duchenne dystrophy is invariably, due to a genetic deletion on the short arm of the X chromosome (Monaco et al., 1986). This gene encodes for the synthesis of a structural protein called dystrophin (Hoffman et al., 1988) which is deficient in a DMD patient. Unfortunately, in spite of this discovery and knowledge, there is still no treatment or cure for muscular dystrophy (Clemens and Caskey, 1994).

Research scientists are presently viewing myoblast transplant (Law et al., 1990; Partridge et al., 1989; Partridge 1991a&b; Gussoni et al., 1992; Huard et al., 1992) and gene transfer (Acsadi et al., 1991; Davis & Jasmin, 1993) as viable, potential therapies. Preliminary research in this aspect seemed successful when myoblast transfer was tried on myopathic mice (Partridge et al., 1989; Karpati et al., 1989) which exhibit a genetic muscle disease similar to DMD in humans. Myopathic mice were used for their similarity to human dystrophy because they also fail to produce dystrophin.

Research of this nature is being extended to human patients (Brooke, 1990; Karpati, 1990; Law et al., 1993; Salvatori et al.,1993; Tremblay et al., 1993; Mendell et al., 1994) but the results are not very promising. The alternate choice of gene transfer therapy holds out greater promise (Karpati and Acsadi, 1993; Vincent et al., 1993; Wolff et al., 1994).

Myoblast transplant, in brief, entails the injection of immature normal muscle cells of a donor, into diseased dystrophic muscle of the patient. It is assumed that transplanted normal myoblasts will fuse with regenerating muscle cells or satellite cells in the patient and, in time, will hopefully replace much of the defective muscle with healthy muscle which would be capable of producing dystrophin. Normal myoblasts are obtained from cultures of normal muscle in a controlled laboratory environment.

Genetic engineering, on the other hand, involves gene transfer via a viral vector (Acsadi et al., 1991; Fardeau, 1993) in the hope that the muscles of the patient may then be able to produce dystrophin which will ultimately lead to the growth of normal muscle cells.

Before these therapies can be adopted, many scientific and practical questions need to be answered, which include methods for growing enough normal cells in the laboratory, the success of gene transfer using viral vectors, the possibility of treating all affected muscles, the problem of rejection, the identification of the precise role played by dystrophin and dystrophin related proteins in the muscle cells and above all, a better understanding of the process of muscle regeneration.

1.2 Statement of the problem

Whatever therapy may be found in the future for muscular dystrophy, it must, of necessity, depend on the regenerative ability of skeletal muscle. Therefore, this research project was undertaken to shed more light on the mechanisms involved in the process of muscle regeneration, and to clarify some points of conflict that exist in this area, especially the identification of origin of precursor cells or myoblasts during regeneration. That there is conflict, is shown clearly by Sloper and Partridge (1980) who concluded that: ***"The source of the mononuclear muscle cell precursors is still uncertain. The idea that an undifferentiated, as it were embryonic, satellite precursor cell can persist through adult life, lying between plasma and basement membranes of the muscle fibre, has gained wide acceptance; but it has not entirely superseded the view that myoblasts can arise by segregation of differentiated myonuclei. It remains possible too that local connective tissue cells and, again, circulating cells may have an accessory role in myogenesis."***

Up to the present time, many scientists, (Reger and Craig, 1968; Shafiq et al., 1968; Church, 1969; Allbrook et al., 1971; Moss and Leblond, 1971; Snow, 1977a&b/1978/1979; Allbrook, 1981; Campion, 1984; Dubowitz, 1985; Carpenter, 1990) in the field of muscle regeneration, believe that satellite cells found in skeletal muscle are responsible for multiplying and giving rise to new muscle.

There are other researchers (Maximow and Bloom, 1944; Hay, 1959/1979; Lee, 1965; Mastaglia and Kakulas, 1969; Reznik, 1969/1976; Teravainen, 1970; Walker, 1972) who suggested that even the myonuclei within the mature skeletal muscle fibre may give rise to new muscle during the regenerative process.

In the course of becoming proficient in culture techniques, my observation of muscle explants maintained under culture conditions for a period of six weeks, led me to believe that during muscle regeneration, sources other than only satellite cells, must give rise to new myoblasts during regeneration. The belief was based on the observation of the large number of cells that continuously migrated or sprouted out from the muscle explants maintained in culture for six weeks. It was difficult to accept that the large numbers of cells arose only out of satellite cell multiplication, knowing that satellite cells constituted only a very small percentage, 4-7% of muscle nuclei (Wakayama and Schotland 1979) of the skeletal muscle. Some workers (Reznik 1969) suggested even lower figures of 0 to 1% for satellite cells in mature muscle.

1.3 Objectives

Therefore, with the design of this project using muscle explants in culture, the hypotheses whether myoblastic precursor cells originate from satellite cells or myonuclei was tested, and their progression to the formation of multinucleate myotubes was traced.

The method, in brief, entailed culturing of muscle explants in a controlled laboratory environment for 10 days. Muscle specimens, for electron microscopy, were removed from cultures on 10 consecutive days. The pre-incubation muscle specimens served as controls. The progressive ultrastructural changes that occurred in the muscle explants, on a day to day basis for 10 days during regeneration, were thus ascertained.

Earlier, ***in vivo*** regeneration studies on animals used injured or minced muscle implants. These experimental designs were disadvantaged by the presence of blood clots, poor perfusion, necrotic debris and outside elements such as fibroblasts and phagocytes which hindered recording of distinct early morphological changes in the regenerating implants. The study of regeneration using muscle explants, in vitro, overcame much of these disadvantages (Askanas, 1979).

This regeneration study was conducted on normal hamster and human skeletal muscles. To the best of my knowledge, ultrastructural investigation of skeletal muscle regeneration in muscle explants in culture, traced on a daily basis for a period of 10 days of incubation, has not been performed by any other researcher before.

As anticipated, this research project revealed positive electron microscope evidence to clarify the main areas of conflict, i.e.:

a) The origin of myoblasts during regeneration in hamster and human skeletal muscle explants.
b) The behaviour of myonuclei during muscle regeneration.
c) The role of phagocytes during muscle regeneration.

At the same time, other ultrastructural changes observed in the cultured explants were recorded and interpreted. Some of the astounding observations and the provocative interpretations appear to go against the dictum of biological sciences, in the field of cell division and new cell creative ability of regenerating muscle.

It is hoped that the information gained on muscle regeneration in explants will help towards a better interpretation of the morphological changes observed in the muscle of experimental subjects undergoing therapeutic trials and in pathological muscle from patients. Furthermore, if the results of this study on regeneration are confirmed in the future at other research stations, the researchers involved in the search for a cure will have to reorientate their therapeutic design and targets. In so doing, the results of the current study will assist in the future management and treatment possibilities of muscular dystrophy, where myoblast transplant or gene transfer methods are contemplated.

An overview of Duchenne muscular dystrophy and the current research status thereon is incorporated as a prelude to the literature review on muscle development and regeneration. This is done intentionally for the benefit of

the readers who may feel inclined to join the battle against muscular dystrophy. Furthermore, the materials used and methods adopted in the current study, are presented in detail to foster the establishment of more muscle culture laboratories in the country.

Ethical clearance for the study was obtained from the Ethical Committees of the University of Natal and the University of Durban-Westville.

CHAPTER 2

REVIEW OF LITERATURE

2.0 INTRODUCTION

Muscular dystrophy provides the background to this thesis on muscle regeneration, hence, a brief overview of this disease and research status to date is presented as a prelude to the review on muscle regeneration. Although much of this overview on muscular dystrophy appears technically irrelevant to the current thesis, it is presented in order to highlight the gravity of the problems confronting those who are afflicted by the dreaded disease, and those researchers engaged in trying to overcome its hazards. Therefore, due to the deep commitment in research in the area of muscular dystrophy, the overview is presented regardless of the work involved in its preparation in the hope that future readers of this thesis may be attracted to research in any of the fields concerning muscular dystrophy other than muscle regeneration alone.

This is followed by pertinent literature review on the development of skeletal muscle, muscle regeneration techniques and revelations in muscle regeneration research.

2.1 MUSCULAR DYSTROPHY

By definition, muscular dystrophy is a generic description for a series of diseases characterised by progressive degeneration of skeletal muscle (Mrak,

1985). The muscle fibres at first attempt to regenerate, but fail to keep pace with the degeneration process. Over the years, muscle in some patients is replaced increasingly by connective and adipose tissue. This often causes the muscle of the patient to enlarge or hypertrophy, the condition being referred to as pseudohypertrophic muscular dystrophy, commonly known as Duchenne muscular dystrophy (Walton, 1988). In other types of muscular dystrophy, the muscle undergoes gradual reduction in size mainly due to the death of the muscle cells, leading to muscle wasting.

2.1.1 Historical background and classification

The distinction of the disease being myopathic and not neuropathic in origin was first established in 1891 (cited in Adams, 1975). In those early days, the disease was thought to be due to poor blood supply to the muscle. The ischaemic condition supposedly caused a short supply of nutrients or trophic factors to the muscle and hence the name 'dystrophy' given to the condition.

The classification of dystrophy is based on criteria such as the mode of inheritance of the defect, the clinical distribution of symptoms in the different muscle groups in the body, and the clinical severity of the disease (Pearson and Young, 1993).

The major muscular dystrophies are Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), Limb girdle muscular dystrophy (LGMD), Fascioscapulohumeral muscular dystrophy and myotonic muscular dystrophy. Of the muscular dystrophies, DMD is the most common and severe type of human dystrophy with the incidence of 1 in 3500 live male

births in all populations (Emery 1980; Gorospe and Hoffman, 1992; Clerk et al., 1993; Smith and Schofield, 1994).

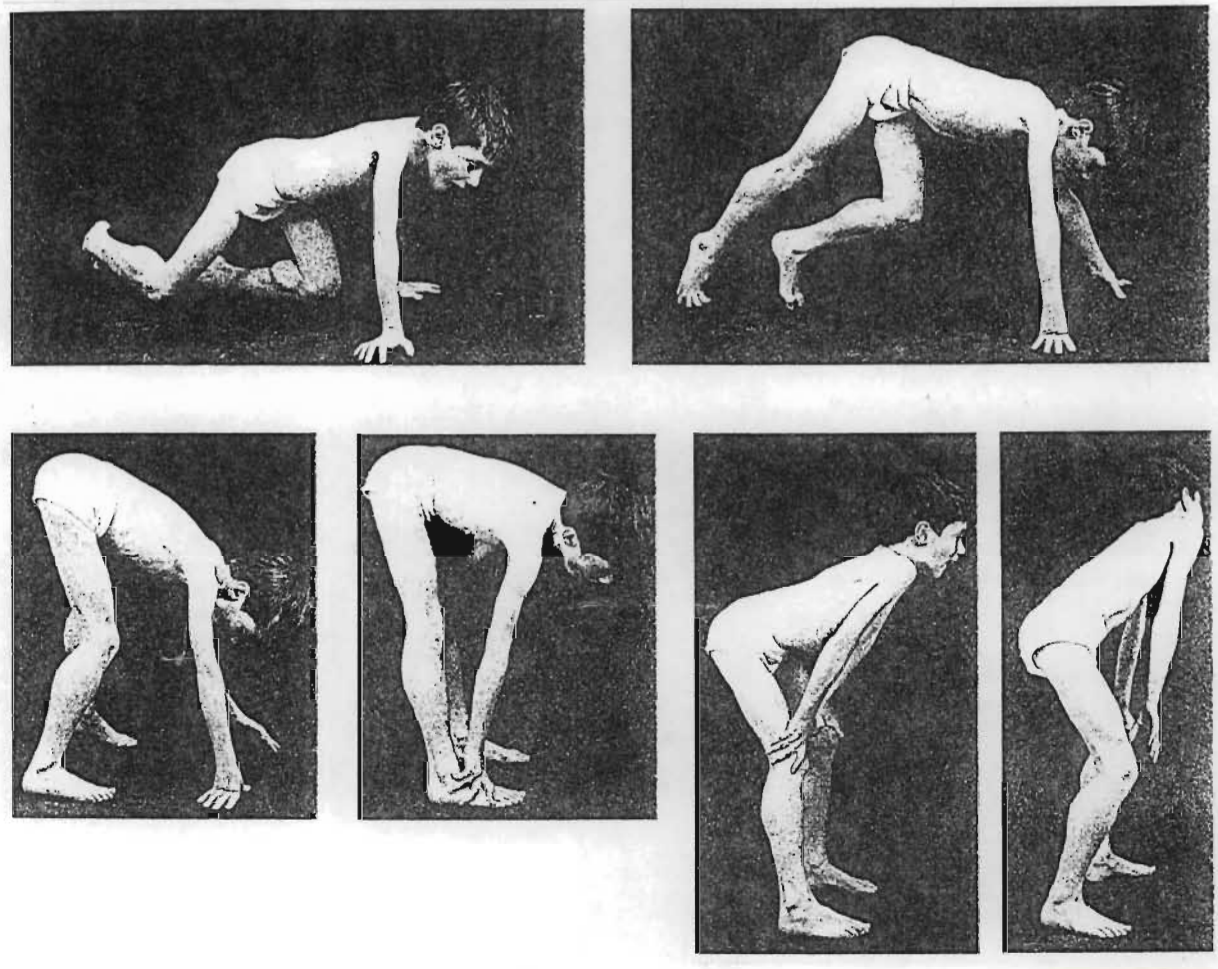
Duchenne type dystrophy was named after Guillaume Benjamin Amand Duchenne who described the muscular dystrophy in question, in 1861 and 1868 (cited in Emery, 1993). Emery also stated that there was controversy in that there is evidence that the disease DMD was first described by an English physician called Edward Meryon in 1851. In another circle, (Bonduelle, 1990) it was felt that F.A. Aran in 1850 gave the first clinical description, synthetic presentation and appellation of 'progressive muscular dystrophy'.

According to Walton and Gardner-Medwin (1988), one third of DMD cases were inherited from multigeneration families, one third from new mutations arising in the mother and the balance as new mutations in isolated males.

2.1.2 Symptoms and diagnostic features of DMD

A description of DMD given by Gowers in 1879 (cited in Dubowitz, 1989b) is as follows: ***"This disease is one of the most interesting, and at the same time most sad, of all those with which we have to deal; interesting on account of its peculiar features and mysterious nature; sad on account of our powerlessness to influence its course, except in very slight degree, and on account of the conditions in which it occurs. It is a disease of early life and early growth. Manifesting itself commonly in the transition from infancy to childhood, it develops with the child's development, grows with its growth - so that every increase in stature means an increase in weakness, and each step takes him a***

year further on the road to helpless infirmity, and in most cases to an early and inevitable death."

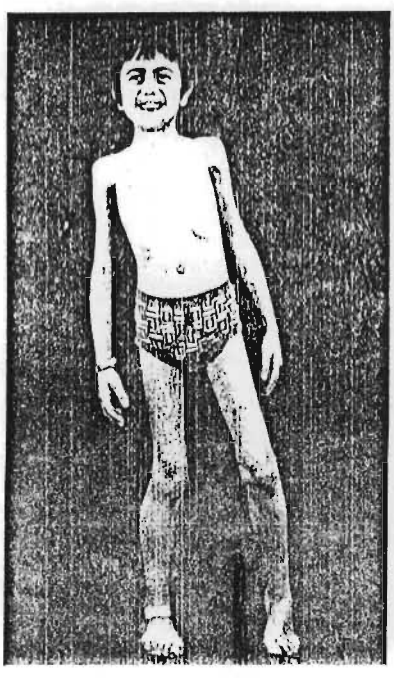


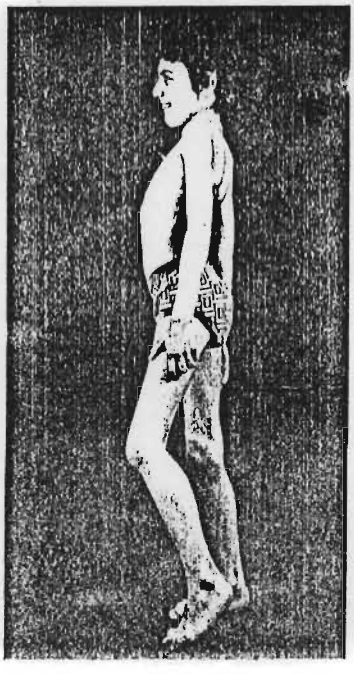
Gower's manoeuvre

DMD like the milder form BMD is inherited as a sex-linked recessive condition. With rare exceptions, it affects mainly males. The onset of the disease usually corresponds in time when the youngster begins to walk, which time, in most cases, is delayed to 18 months. Between 3-5 years the calf muscles show signs of hypertrophy, and the heels are raised so that the infant walks on toes. They often trip and fall, even on flat surfaces. When down they have tremendous difficulty getting up. The hands are placed on

the ground for support and balance to assist with rising which has a characteristic pattern described as Gowers manoeuvre (see illustrations above: taken from Dubowitz, 1989).

They walk with a waddling gait being unable to jump or climb stairs (Anderson and Kunkel 1992) as shown in the diagram below (Dubowitz, 1989).





waddling gait

scoliosis

To balance while walking, they are forced to push out their chests as if they suffer lordosis. Before they become wheelchair-bound at about 9 years, they have to hold on to rails or helpers to get around. The age when ambulation is lost is one of the milestones in the progression of DMD (Nicholson et al., 1993). In the few years while in the wheelchair, their muscles undergoing progressive degeneration, causes them to sit with a

slumped posture so much so that they soon develop scoliosis. They are soon lost to the world before the second or early third decade, as a result of respiratory or cardiac failure.

In a study by Biller et al. (1987), it was found that 83% of the patients with DMD and 50% with BMD had electrocardiographic abnormalities. According to Saito et al. (1994), death due to respiratory failure is more common in DMD patients while death due to a cardiac involvement is more often associated with the BMD patients.

Short stature was reported as a characteristic feature of DMD patients in a growth study (Eiholzer, 1988). The academic achievement of many of the DMD cases is impaired (Anand, 1983; Mrak, 1985; Gorospe and Hoffman, 1992; Pearson and Young, 1993). This may possibly be attributed to a deficiency of the dystrophin isoform found in brain tissue (Lidov et al., 1993), but Rowlands (1988a) reported that there was no consistency between mental retardation and the diseases that were encoded at Xp21. Whether poor academic achievement was due to the genetic disorder or simply because the patients were not motivated enough to perform in the face of more serious issues of psychological and physical trauma were questions that needed some attention!

Most consistent findings in histological inspection of biopsies from DMD patients included muscle degeneration with pyknotic clumps of nuclei, central nucleation, fibre splitting and increased endomysial and adipose tissues (Dubowitz and Brooke, 1973; Thompson et al., 1983). Recently D'Amore et al. (1994) suggested that basic fibroblast growth factor, which was found to be elevated in a number of DMD subjects, to be a possible

factor contributing to the increased fibrosis and muscle weakness that prevailed in DMD. Fibroblast growth factor supposedly stimulated connective tissue growth and suppressed skeletal muscle differentiation.

2.1.3 Early research

DMD being the most common and the severest type of dystrophy, was also the most researched dystrophy. The research impetus into DMD was due to the notion that if a solution to the problem of DMD was obtained, then solutions to many of the other genetic diseases would follow.

Extensive research was and is still being promoted financially by the many Muscular Dystrophy Associations around the globe from funds contributed by the generous public.

There were many hypotheses on the aetiology of the disease in the past when research was centred on:

i) a defect in cell membranes (Lucy, 1980; Rowlands, 1980; Jones and Witkowski, 1981/1983; Mokri and Engel, 1975),

ii) a primary lesion of the motor neurones (Dubowitz, 1973),

iii) a lesion in the muscle microcirculation and basement membrane abnormality (Lipton, 1979; Fidzia'nska et al., 1987; Miike et al., 1987)

iv) and, an anomaly of the connective tissue, with most of the evidence favouring the first possibility (Yasin et al.,1979).

Evidence of high levels of certain serum enzymes, especially creatine kinase (Moosa 1982), and increased intracellular concentration of calcium ions in

muscular dystrophy (Lucy 1980; Emery 1980) suggested the presence of 'leaky' muscle membranes in DMD. Alongside all this research mentioned above, muscle regeneration studies discussed later in the chapter were also conducted.

Structural and biochemical studies on skeletal muscle itself have been augmented with the advent of improved techniques to culture both normal and diseased skeletal muscle (Konigsberg, 1963; Bishop et al., 1971; Yasin et al., 1977/1982). Generalised membrane defect was not indicated in later studies using cultured skeletal muscle and skin fibroblasts from DMD patients (Mongini et al., 1988). In another study using cultured cells, it was tentatively concluded that the activity of the enzyme acetylcholinesterase was impaired in myotubes of dystrophic patients. Freeze-fracture studies of the plasmalemma in mdx mice demonstrated ultrastructural abnormalities similar to those in human DMD (Shibuya and Wakayama, 1988).

2.1.4 Recent and current research

In the 1980's research concentrated on the genetic aspects of the disease. The aetiology of DMD remained elusive until recently. Two marker genes were isolated which were co-inherited with DMD 90% of the time. With linkage analysis, these were localised to be on the short arm of the X chromosome in the band Xp21 (Murray et al., 1982; Davies et al., 1983; Bakker et al., 1985; Monaco et al., 1986). This discovery was made in females (see diagram below) who exhibited a dystrophy similar to DMD in boys, the cause in these cases being translocation of the DMD gene from the X chromosome to autosomes. The break points in the X chromosome were at locus Xp21 (Burghes et al., 1987; Dubowitz, 1989).

Female with DMD gene translocation abnormality assisted with callipers (Dubowitz, 1989).

Initially a large protein, nebulin was thought to be the protein involved in the pathogenesis of DMD. Evidence in subsequent studies precluded the possibility that nebulin was the DMD gene product (Bonilla et al., 1988; Pernelle et al., 1988)

Many inroads have since been made into the fundamental understanding of the disease. Analysis of DMD and BMD gene deletions were published in 1986 by Kunkel. Soon the complete cloning of the very large 14 kb DMD gene was achieved (Koenig et al., 1987). By the use of polyclonal antibodies, the DMD gene product, a protein named dystrophin which was absent or deficient in DMD patients, has now been identified (Hoffman et al., 1987a&b). Clinical concepts about the myopathies were inevitably revolutionized with the advances in molecular genetics (Rowlands, 1988a&b; Stedman and Sarkar, 1988).

2.1.4.1 Dystrophin

The discovery of the DMD gene and its product, dystrophin a large protein molecule (427 kD), named by Kunkel and his associates in 1987, opened the flood gates to research in pursuit of gene identification of other genetic diseases (Payne and Roses, 1988). The search for therapies for muscular dystrophy, using the information on dystrophin, is currently being vigorously researched (Hoffman et al., 1988; Chamberlain, 1991; Ohlendieck and Campbell, 1991; Wessels et al., 1991; Tidball et al., 1992; Nicholson et al., 1993a&b; Law and Tidball, 1993; Padberg, 1993; Lidov et al., 1993; Helliwell et al., 1994; Matsumura et al., 1994; Sewry et al., 1994).

Dystrophin was initially thought to be located in triadic junctions of muscle (Hoffman et al., 1987) but subsequent research showed that dystrophin was located on the inner cytoplasmic surface of the basement membrane (Watkins et al., 1988). The presence of dystrophin was more prominent in the surface membranes of intrafusal muscle fibres and neuromuscular junctions than that found in skeletal and cardiac muscle fibres (Miyatake et al., 1989). Miyatake et al. (1989) also localized dystrophin in smooth muscle of viscera including blood vessels.

Dystrophin was also identified in cultures of normal human muscle, but not in the muscle of DMD patients (Ecob-Prince et al., 1989). Myofilaments assembled and functioned normally in DMD muscle, but it was believed that dystrophin deficiency lead to the clinical weakness by causing breakdown of the myofibres that were once capable of generating normal force (Horowits et al., 1990). Normal thin filaments associated with myotendinous junctions were attributed in part to normal dystrophin presence (Tidball and Law, 1991).

Some of the other revelations include that dystrophin was a relatively scarce protein in the cell, making up about 5% of the total plasmalemma associated cytoskeletal protein (Ohlendieck and Campbell, 1991). Previously, it was believed that dystrophin was present all along the sarcolemma, but Porter et al. (1992) suggested that dystrophin was localized to the I-bands and M-lines only. Dystrophin was thought to anchor the contractile elements to the surface membrane. This was supported by Law and Tidball (1993).

Increased intracellular calcium concentrations, as a result of membrane instability in muscular dystrophy, was implicated in promoting cell protein degradation and necrosis in muscular dystrophy (Glesby et al., 1988; Turner et al., 1988). Dystrophin deficiency and increased cellular calcium concentrations coupled with metabolic disturbances in the muscle of dystrophic patients was probably attributed to increased levels of oxidative stress. The effect of oxygen free radicals was suggested as the fundamental basis of many of the disease characteristics of muscular dystrophy (Baker and Austin, 1989; Murphy and Kehrer, 1989).

Karpati (1992) reported the absence of dystrophin in most of the skeletal muscle fibres of DMD patients and that variable deficiencies were found in BMD. On the other hand, according to Yau et al.(1993) one third of the DMD and BMD patients did not show gross deletions of the dystrophin gene and that this made prenatal diagnosis and carrier detection a problem.

Dystrophin, as an anchor protein, functions in association with glycoproteins to link the subsarcolemmal cytoskeleton to the extracellular matrix. Four glycoproteins were found to be integral components of the dystrophin complex with one of them being greatly reduced in DMD patients, and this

was suggestive of the first stages of the molecular pathogenesis of muscular dystrophy (Ervasti et al., 1990). The absence of dystrophin accompanied by decreased dystroglycans or dystrophin associated glycoproteins (DAG) was suggested as the reason for membrane instability and necrosis in DMD muscle (Helliwell et al., 1994; Matsumura et al., 1994). Some rare cases, exhibiting DMD symptoms, showed decreased DAG expression even though there was no deficiency of dystrophin (Sewry et al., 1994). The DAGs apparently are expressed only in striated muscle and may therefore be important in the pathogenesis of DMD (Yamamoto et al., 1994).

The dystrophin-associated protein (DAP), called utrophin, were apparently increased in such cases. Tinsley and Davies (1993) suggested the possibility that utrophin may have the capability of performing the same cellular functions as dystrophin. They went on to state that if that was the case, it might be possible to regulate utrophin production as an alternate route to dystrophin gene therapy as an answer for DMD and BMD.

Even though the underlying biochemical defect in DMD was a deficiency in dystrophin, there was no obvious temporal correlation between dystrophin deficiency with the progressive histopathological, as well as with the clinical features of the disorder. The defect in DMD is present from fetal life and yet the clinical picture presented itself much later, progressing rapidly thereafter (Gorospe and Hoffman, 1992). Nerve-muscle co-cultures performed on muscle biopsies from DMD patients showed some dystrophin positive fibres. This positive showing of dystrophin was thought to be due either to clonal selection of dystrophin positive fibres observed in one of the biopsies or due to a "frame restoring" mutation that might have occurred under culture conditions (Fanin et al., 1993).

2.1.4.2 Myoblast transplant and gene transfer therapy

Now that dystrophin and DAG deficiency in DMD has been confirmed, the problem that faced researchers was to find a way of conveying these muscle proteins into the muscle of patients (Blau, 1993; Cullen et al., 1994). Unfortunately these proteins, being structural elements of the sarcolemma and not found in the blood, can not be administered or conveyed to the muscle therapeutically by means of simple injections into the patient. The two avenues presently researched to convey these proteins into dystrophin deficient muscle are myoblast transplant or gene transfer methods (Chamberlain, 1991).

2.1.4.2.1 Myoblast transplant

Myoblast transplant involved the injection of normal mononucleated muscle precursor cells, harvested in clonal cell cultures in the laboratory, into dystrophic muscle in the hope that these normal cells will fuse with the sick host cells. In this way, the dystrophic genome may be admitted into the host cells to start producing dystrophin to overcome the deficiency (Partridge, 1991a; Fardeau, 1993). Experiments were initially performed with some success in animal models using the mdx mice. These mice also exhibit the dystrophic gene defect and deficiency in dystrophin like the human DMD. In these transplant experiments some mdx myofibres were converted from dystrophin negative to dystrophin positive fibres (Chamberlain et al., 1989; Karpati et al., 1989; Partridge et al., 1989).

Karpati (1990) suggested that for transplant therapy to be applied to the human situation, it must satisfy conditions of feasibility, safety and that it

must be efficacious. These transplant techniques were then extended to human trials but without resounding success (Brooke, 1990; Law et al., 1990a&b/1991/1993; Huard et al., 1992; Karpati et al., 1993; Morgan, 1994). Many obstacles were identified with the therapeutic application of the myoblast transplant to the human disease (Partridge, 1991a; Roy et al., 1993).

There was dissent among the research fraternity (Thompson, 1992). It was felt that it was unethical to continue myoblast transplant tests on human patients until the success of such therapeutic applications were problem free. These tests must first be tried with unquestionable success in animal models that closely resemble the human DMD situation. Presently, the best models would be the canine ones (Cooper et al., 1988; Sharp et al., 1993). The golden retriever dogs suffer from a dystrophy similar to human. The difference between the canine and the mice models was that the canines, like the human, lose ambulation with the progression of the disease. The mdx mice on the other hand do not lose ambulation nor do they display gross muscle weakness, and this perhaps is due to the fact that these animals, for some unknown reason, exhibit continued regeneration of muscle even in the adult stage.

The harmful effects of immunosuppressive drugs and general anaesthesia on the patient, compared to the dismal result obtained in the transplant method, did not justify the risk to the patients. Very little functional capabilities were improved or achieved with no change in the ambulatory status of the patient (Mendell et al., 1994). Even if consent was obtained for such trials, it was felt that the young boys and their parents, with all the emotional implications of having DMD, were in no position to give true

informed consent. However, these criticisms did not deter the researchers involved as they felt 'The initial lack of success in an experimental work is no justification to advocate its cessation' (Karpati cited in Thompson, 1992).

Even if preliminary results on myoblast transplant showed promise, it was bound, ultimately, to prove to be an impractical method of treatment of dystrophy. The transplanted myoblasts will be localized and will benefit only the muscle that is injected with the donor cells. The donor myoblasts do not migrate from muscle to muscle. For success with this method, it will require that every skeletal muscle in the body be injected with these normal or genetically normalised cells. This was virtually impossible! Satoh et al. (1993) clearly demonstrated in their work using mdx mice that the injected donor myoblasts remained near the injected site and that there was a poor dystrophin positive response in the muscle fibres. The editorial caption, "Myoblast transfer in muscular dystrophy: panacea or pie in the sky" says it all (Dubowitz, 1992).

2.1.4.2.2 Gene transfer therapy

The more promising method of treatment in the future for muscular dystrophy was offered by the gene transfer method (Ascadi, 1994; Karpati, 1994). The gene to be transferred can be introduced to target cells, ***in vivo***, or the gene may be introduced to target cells, ***in vitro,*** and then reintroduced to the tissues (Karpati and Ascadi, 1993). Direct transfer of gene into the extracellular space of the muscle was conveyed by viral vectors such as retrovirus and adenovirus. But the administration of viral vectors needed to address the problems of toxicity, immunoreaction and, ***in vivo,*** recombination of the gene constructs (Davis and Jasmin, 1993;

Karpati and Ascadi, 1993; Ascadi, 1994). On the other hand, Vincent et al. (1993), having used the adenovirus mediated transfer method of a human dystrophin minigene in their work with mdx mouse, reported long term correction of dystrophic degeneration of mutant mouse deficient in dystrophin.

Transduced normal human satellite cells with retrovirus, injected into regenerating muscle of immunodeficient mice, formed new fibres which revealed the product of the reporter gene for two months after injection. From these findings Salvatori et al. (1993) concluded that the human-mouse model allowed ***in vivo*** testing of similar gene therapy approaches. Transgenic techniques used by Lee et al. (1993) achieved regional restoration of recombinant dystrophin to the muscle of the mdx mouse and regional restoration of normal muscle morphology. Their study indicated a correlation between the level of muscle fibers expressing recombinant dystrophin and the level of muscle fibers with peripheral nuclei. In another study it was shown that transgenic induction of dystrophin in mdx mice overcame the morphological and immunohistological symptoms of muscular dystrophy, providing functional evidence for the feasibility of gene therapy (Cox et al., 1993). Research in this area of gene transfer is ongoing with the use of animal models. When success is achieved, it is hoped that this method of therapy will work for the neuromuscular diseases in human.

2.1.5 Interim treatment and support therapy

The ultimate requirement from research would be that it provides answers to prevent the progression of the neuromuscular disease or help to cure the disease altogether. In the absence of such treatment to date, prevention of

the disease relies on the identification of carriers and on prenatal diagnosis using amniocytes (Simard et al., 1991) and/or fetal cells (Evans et al., 1993). Diagnostic methods are available for such early detections of the disease (Goldblatt et al., 1987; Payne and Roses, 1988; Simard et al., 1991). The only problem with the diagnostic methods is that they become limited by the high incidence of spontaneous mutation rate of the DMD gene. The occurrence of the disease by spontaneous mutation in such individuals is unpredictable and, therefore, requires new strategies for detection and treatment (Anderson and Kunkel, 1992).

2.1.5.1 Treatment with chemical agents

Some of the chemical gents used to alleviate the DMD patients included calcium antagonists such as diltiazem. The results from manual muscle tests and functional activity using diltiazem as a chronic treatment suggested a beneficial effect to DMD patients (Bertoni et al., 1988). Johnson and Bhattacharya (1993) supported this with their findings on dystrophic hamsters. In their work they examined the effect of diltiazem and other calcium channel blockers on the regulation of excessive intracellular calcium accumulation. It is well documented that the intracellular calcium concentration is elevated in DMD muscle (Palmieri, 1993). Calcium concentration was also found to be elevated in platelets, erythrocytes and fibroblasts (Moses et al., 1990).

Prednisone treatment of dystrophy was reported to improve muscle strength and function, but the mechanism is not known (Burrow et al., 1991). Kissel et al. (1991), from their investigations, suggested that immunosuppressive effects assisted in mediating this improved muscle strength in DMD patients.

However, in a recent study, Kissel et al. (1993) concluded that immunosuppressive actions were not the primary mechanism of prednisone-induced clinical improvement. Prednisone tried on mdx mouse also supported its beneficial effects on improving strength and endurance in DMD mouse (Hudecki et al., 1993). A dramatic case was recorded recently in a boy of 4 years who exhibited most of the clinical symptoms characterising DMD. When treated with prednisone, he started running and climbing stairs, all that he could not do before, just 24 hours after treatment. He had normal dystrophin and the aetiology of this condition was unknown. With this experience, a case was made for the treatment with prednisone for all undiagnosed progressive myopathies (Bradley et al., 1994). Angelini et al. (1994) tried deflazocort in DMD patients as a long term treatment and reported improved motor capabilities in the patients and prolonged ambulation for a little over a year. The side effects recorded were mild, which included weight gain and behavioural changes.

2.1.5.2 Surgery, exercise and support equipment.

Apart from the very important psychological support necessary for the dystrophic patients, there were many ways in which some measure of comfort could be given to the patient. Surgical intervention was sometimes advised to reduce early contractures or shortening by tenotomy (Dubowitz, 1988). Freeing the Achilles tendon helped keep the patients on their feet for a little while longer (Fenichel and Robison, 1988). In order to avoid scoliosis, which became progressively worse when the patients stopped walking, routine spinal arthrodesis was to be given consideration (Brooke et al., 1989; Smith et al., 1989).

Leg braces, walking aids and night splints may be used to keep the patients ambulant for as long as possible and out of the dreaded confines of a wheelchair. Support jackets may be worn to afford comfort while wheelchair bound.

Walking and breathing exercises were advocated. Disuse of the muscles only helped to debilitate the patient further by promoting muscle weakness, decreased cardiovascular performance and promoting contractures (Fenichel and Robison, 1988).

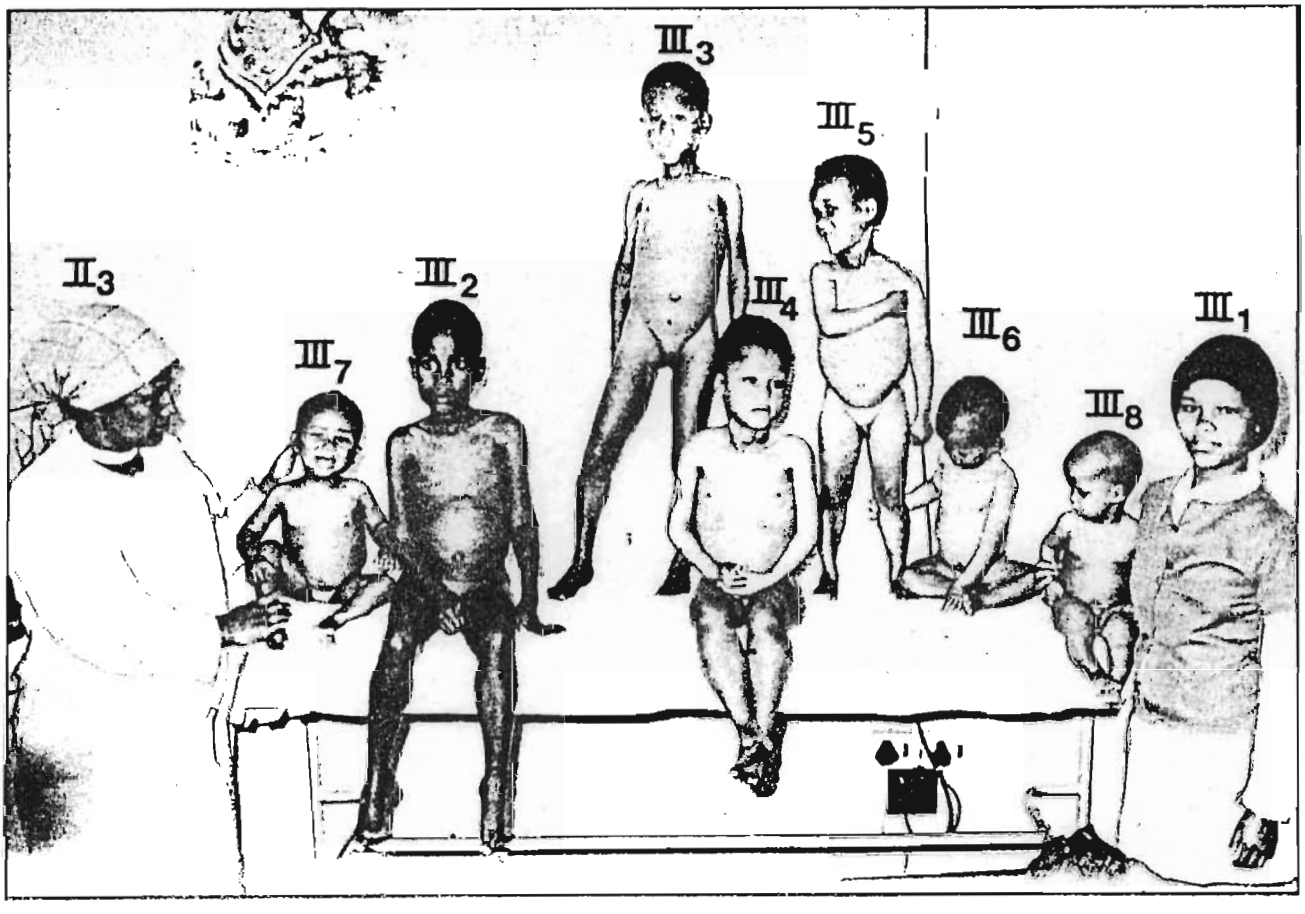
Low frequency electrical treatment, having shown some benefit in improving muscle contraction in young ambulant children with DMD, was also suggested as possible therapy (Dubowitz, 1988; Milner-Brown and Miller, 1988; Scott et al., 1990).

2.1.6 Diagnostic methods.

The need for proper and early diagnosis cannot be over-emphasised as shown in the diagram on the following page.

Procedures helpful in diagnosing muscle dystrophy include:

(i) familial history
(ii) clinical examination
iii) biochemical tests on blood and muscle biopsy tissue
iv) electromyography
v) histological and ultrastructural investigation of biopsies
vi) nuclear magnetic resonance and
vii) computerised tomography and ultrasound (Pearson and Young, 1993).



Mother on left with eight children - six of her sons III 2 to III 7 afflicted with muscular dystrophy - could have been avoided with early diagnosis (Moosa, 1982).

Estimation of serum enzyme concentration of creatine kinase were of value as initial laboratory diagnosis especially in the very young patients, as the enzyme concentrations declined with age. Young female carriers frequently exhibited elevated creatine kinase.

Muscle biopsies, obtained as open or needle biopsies (Maunder-Sewry and Dubowitz, 1981), were helpful in making histological and electron microscopic diagnosis. Ultrasound scanning was useful in muscle selection

for needle biopsies. Imaging of muscle by ultrasound was also recommended as a noninvasive and pleasant out-patient procedure, valuable as a screening test in the investigation of neuromuscular diseases (Heckmatt et al., 1988; Lamminen et al., 1988).

2.1.6.1 Precise diagnosis

Today, with the identification of the defective gene assembly on the chromosome, and the identification of the DMD gene product, dystrophin, together with the dystroglycans, correct identification and classification of a number of the neuromuscular diseases are possible. Clinical symptoms and blood chemistry results together with histological diagnosis were not always efficient for the proper diagnosis and classification of the dystrophies as shown for the case of a 4 year old boy (Bradley et al., 1994). Tests for dystrophin, in a number of cases has proved earlier diagnosis to be incorrect. Anomalies in clinical pictures were sometimes presented as in the case of two brothers who had the identical gene defect for BMD (Medori et al., 1989). They were shown to present different clinical courses, one having normal muscle strength at 26 yrs while the other had severe disability at the same age limits. The possible reasons forwarded were that this discrepancy was due to intrinsic muscle factors, to modified genes or internal environmental influences, as the stronger brother was treated with antiepileptic drugs.

In another case, Wilton et al. (1994) highlighted the diagnostic power of precise identification by showing that two boys in a family had DMD, but the mother and their sisters did not carry the mutation. That counselling in this family by conventional belief would have been erroneous. The mutation

in this case was due to gonadal mosaicism. In gonadal mosaicism, gene mutations in the females were said to occur in oogenesis and point mutations in spermatogenesis (Grimm et al., 1994).

2.1.6.2 Prenatal diagnosis

Prenatal diagnosis by DNA analysis of genetic abnormalities using amniocytes and cells of the chorionic villus is today possible using the polymerase chain reaction (PCR) test, but, unfortunately this technique is not informative in all patients. Furthermore, the detection of dystrophin by immunocytochemistry was not possible as these cells do not produce the protein. To overcome this hurdle, myogenesis may be induced in amniocytes and chorionic villus cells in culture, with the use of MyoD, a gene regulating myogenesis in cells. This MyoD was transfected with the retrovirus as vectors. Dystrophin expression could then be identified in these cells by immunocytochemistry (Sancho et al., 1993). Fetal muscle biopsy testing was also recommended for the diagnosis of DMD (Evans et al., 1993). In this way, prenatal diagnosis of DMD could be made with some measure of precision.

2.1.6.3 Carrier and dystrophinopathy

Carriers are now identified with a greater measure of reliability. The efficiency of the tests available at the present time also identifies the many sporadic cases due to spontaneous gene mutation.

The condition in females who were deficient in dystrophin and had dystrophic symptoms was referred to as dystrophinopathy.

Dystrophinopathy was believed to be due to X chromosome inactivation or due to paternal transmission of dystrophin gene mutation for yet unknown reasons (Pegoraro et al., 1994). Yoshioka et al. (1990) reported the incidence of female Duchenne muscular dystrophy, showing deletion of the DMD cDNA within Xp21.

2.1.6.4 Identification of young DMD patients

For the young DMD patient with no family history, a number of laboratory diagnostic tests are available. The use of blood cells is less traumatic than the use of muscle biopsies. The polymerase chain reaction (PCR) requiring blood samples for test, was a cost effective method for routine laboratory diagnosis. The drawback in this diagnostic method as it stands today, is that the prediction level was up to 85%.

The most accurate diagnostic method is the Western blotting technique used on muscle biopsies, the only disadvantages being that it is time consuming and most expensive. The cheaper technique using immunocytochemistry on muscle biopsies was quicker and as effective as the blotting technique for DMD diagnosis. Immunocytochemistry is also useful in identifying between limb-girdle dystrophy and a non-deleted manifesting carrier of DMD or BMD in women. Genetic analysis by PCR and immunocytochemistry is recommended to diagnose between DMD and the autosomal recessive muscular dystrophy. Identification between BMD and the autosomal muscular dystrophy was better served by the PCR and the immunoblotting tests (Nicholson et al., 1993b; Tachi et al., 1993; Richards and Iannaccone, 1994).

2.1.7 *Conclusion*

It must be emphasized that only the salient points have been mentioned in this review on dystrophy with greater emphasis laid on DMD. With the discovery of dystrophin and dystroglycans together with the availability of the diagnostic tests, some relief is obtained in the way of prevention by being able to make prenatal diagnosis.

Volumes of research is currently ongoing for the whole spectrum of neuromuscular diseases. Some of the topics of current research are published in the first Supplement of the journal Muscle & Nerve, 1994, which contains all the abstracts of work presented at the VIII International Congress on Neuromuscular Diseases held in Japan, July 1994.

Gene transfer therapy appeared to hold out the greatest promise of a solution to muscular dystrophy. Researchers hope to overcome the preliminary technical hitches and prove the efficacy of this therapeutic method, using animal models of DMD, in the near future. Until such time, those afflicted with the disease together with their parents and close ones must keep their chin up and pray research finally succeeds.

2.2 DEVELOPMENT OF SKELETAL MUSCLE

In this section, a brief account of the development of mature skeletal muscle from embryonic precursor cells will be discussed.

2.2.1 Embryonic myogenesis of skeletal muscle fibres

In vertebrates, striated or skeletal muscle is derived from the mesoderm in the segment called myotome. Precursor cells of muscle or myoblasts in this segment are initially round forms which subsequently become spindle shaped. These spindle shaped cells, as they increase in number mitotically, align themselves alongside each other as described by Speidel (1938) for the regeneration of skeletal muscle in the tail of living tadpoles. Contact between these spindle shaped cells resulted in cytoplasmic fusion of the cells forming myotubes which are syncytial and multinucleate. A similar process of myotube formation took place when mononucleated myoblastic cells were cultured. The dissociated mononucleated cells were initially round when placed into the culture flasks, and on incubation they formed spindle cells. These spindle cells proliferated, fused with each other and finally formed multinucleate myotubes (see Figs. 1 and 2 C-F).

According to Fischman (1972), cells ready to fuse were considered to be postmitotic. These postmitotic cells, which sometimes displayed early myofibrillar development, were characterised as myoblasts. Mononucleated cells which underwent active mitotic division, and in which myofibrillar development was not present at any stage, were referred to as presumptive myoblasts (Carlson, 1973). In the developing myotubes myofibrils soon made their appearance. Z lines became quite distinct and were evenly spaced at intervals along thickened strands of developing myofibrils. During

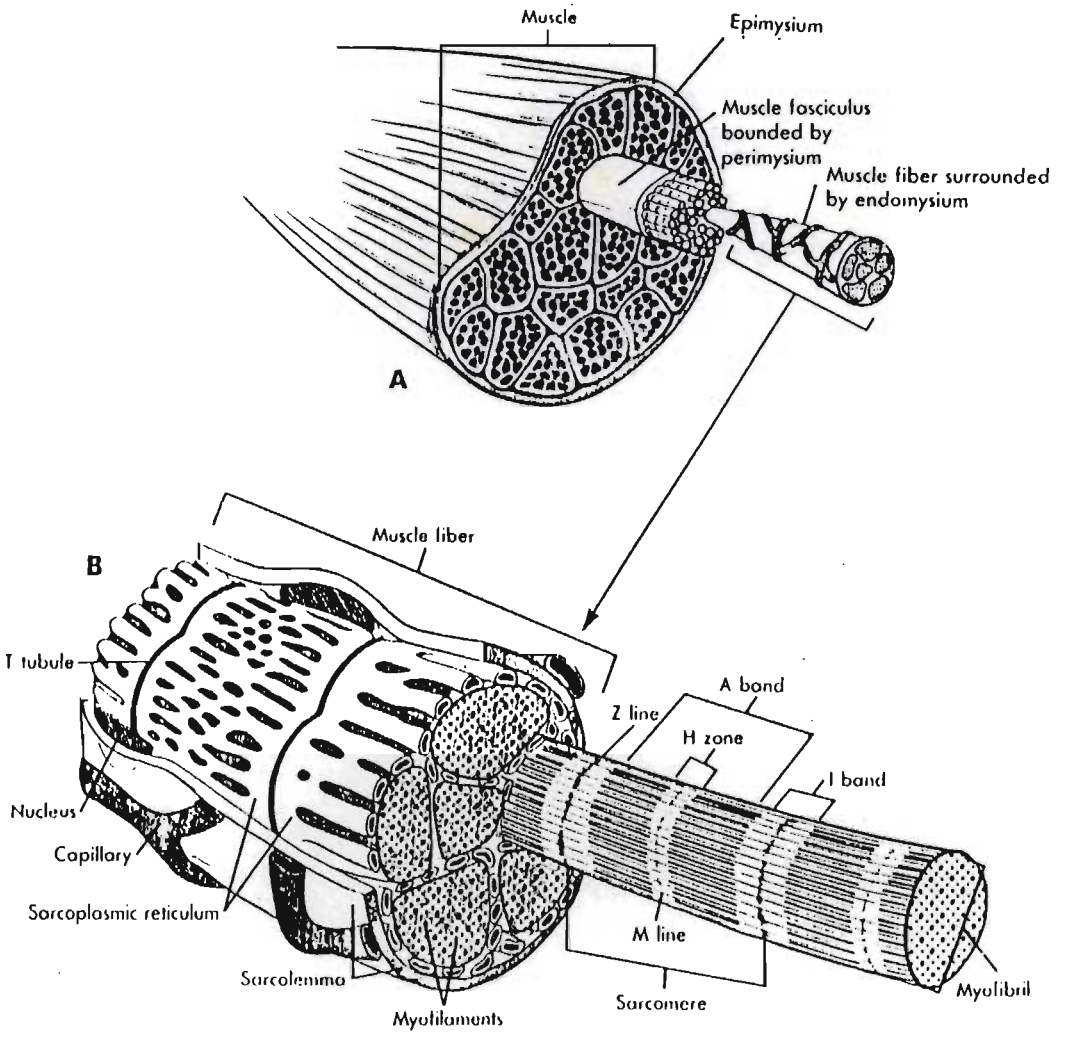
development the nuclei were centrally located, but became peripherally placed as the myotube matured to a fully grown myofibre (Carlson, 1973). This displacement of the nuclei to the periphery under the sarcolemma may be attributed to the filling up of the growing myotube with longitudinal arrays of myofibrils.

It is well documented that the mononucleated myoblasts have all the usual organelles as any other generalized animal cell (Bloom and Fawcett, 1968). The organelles, such as the endoplasmic reticulum and the mitochondria become distributed to specific areas as the myotube proceeds to maturity. The Golgi complex becomes indistinguishable from the endoplasmic reticulum in the myofibre. The nuclei of the mononucleated cells are dense granular often revealing clear nucleoli, usually two in number. Nuclei become euchromatic in the mature myofibre (Dubowitz, 1985).

The myofibres developed in this way were believed to split longitudinally (Schmalbruch, 1976) to increase the number of myofibres making up the skeletal muscle of the developing embryo until approximately 170 mm long (Maximow and Bloom, 1944; Bloom and Fawcett, 1968). Thereafter, future growth occurs to increase only the size of the fibres already present. On the other hand, other researchers believed that the splitting of fibers and formation of new fibers from undifferentiated cells continues in the newborn mammalian muscle (Schultz, 1976; Campion, 1984).

2.2.2 Morphology of mature skeletal muscle

Mature skeletal muscle as an organ is covered by connective tissue sheath, the epimysium. This epimysium, with dense regular collagen fibres, merges



Diagrammatic structures of the skeletal muscle (Moffet et al., 1993).

at either end of the muscle belly with tendons, aponeurosis or the periosteum of bone. Extensions of the perimysium into the muscle belly subdivides the muscle organ into many muscle bundles or fasciculi each covered by thinner connective tissue sheath, the perimysium. Each fasciculus is made up of many myofibres each of which is separated from the adjacent fibre by connective tissue referred to as the endomysium which is continuous with the perimysium. Skeletal muscle fibres are characteristically long (up to 10 cm) and cylindrical fibres with an even diameter (20-50 um) covered by a sarcolemma that separates it from the

external environment. The myofibres are characteristically unbranched.

Electron micrograph of skeletal muscle fibre showing A and I bands, terminal triads Td (T tubule & terminal cisternae TC), sarcoplasmic reticulum SR, mitochondria M (Burkitt et al., 1993).

2.2.2.1 Sarcolemma, basement membrane and plasmalemma

The sarcolemma is made up of the basement membrane on the outside and the plasmalemma on the inner surface. These two layers are closely applied. The basement membrane is secreted by the muscle fibre itself and is said to be composed of laminin, collagen, glycoproteins, proteoglycans and fibronectin. The plasmalemma being the true limiting cytoplasmic membrane of the muscle fibre is the electrically excitable envelope composed of lipid bilayer and structural, receptor and metabolically active proteins (Dubowitz, 1985).

2.2.2.2 Myonucleus

The myofibres are multinucleate with elliptical shaped nuclei in longitudinal sections (Dubowitz, 1985). The multinucleation was generally attributed to many mononucleated cells fusing to ultimately form mature myofibres (Bloom and Fawcett, 1968). The myonuclei are characteristically located at the periphery under the sarcolemma. The normal myonuclei sometimes revealed a prominent nucleolus and finely stippled nucleoplasm usually being described as euchromatic with a thin peripheral rim of dense chromatin (Dubowitz, 1985).

2.2.2.3 Myofibrils, actin and myosin filaments

The myofibres have clear and prominent striations due to the presence of actin and myosin filaments of the myofibrils. The thin actin filaments with the Z line in the centre, scattering light evenly make up the I (isotropic) band and the thicker and darker myosin filaments make up the A (anisotropic)

band. Hundreds to thousands of myofibrils compose the main body or volume (85-90%) of the myofibre sarcoplasm. Each myofibril is separated from the next by intermyofibrillar space which houses the subcellular constituents such as mitochondria and sarcoplasmic reticulum.

2.2.2.4 Mitochondria and sarcoplasmic reticulum

The mitochondria are found in the subsarcolemmal region usually in proximity to myonuclei, and are generally round to ovoid. Those found in the intermyofibrillar space, sandwiched in between the myofibrils, often appear longitudinal. Regions in between the membranous cristae contain amorphous material. Mitochondria in the intermyofibrillar space, viewed in EM sections of muscle, are usually prominent in the regions adjacent to the I bands.

The sarcoplasmic reticulum in muscle fibres is derived by invagination of the sarcolemma (Ezerman and Ishikawa, 1967) forming a central tubule. The central tubule enters the myofibre more or less at right angles at the points where the Z discs are in register and branches to move around the Z disc giving the so called T-tubule formation as seen in frog skeletal muscle (Ham, 1965). With the EM, in longitudinal sections of human muscle, the pale centrotubule is identified in the region of the A/I band interface accompanied on either side by dense terminal sacs of the sarcoplasmic reticulum forming the cytoplasmic 'triad' (Dubowitz, 1985).

2.2.2.5 Ribosomes, lipid droplets and glycogen

The sarcoplasm also contains the ribosomes, lipid droplets, and glycogen

granules. The ribosomes are smaller and more electron dense than the glycogen granules (Mair and Tome, 1972).

2.2.2.6 Intermediate filaments and microtubules

Intermediate filaments occur mainly in the subsarcolemmal regions of sarcoplasm. The proteins of these filaments are immunologically distinct from other filament proteins in the fibre. These are desmin and vimentin (cited in Dubowitz, 1985). Desmin is localized at the periphery of the Z line providing an interconnecting network linking myofibrils with each other and with the sarcolemma, as well as the Z line with the nuclear membrane (Tokuyasu et al., 1983). Vimentin was shown to be associated with the nuclear membrane and with the sarcolemma. Dubowitz (1985) suggested that vimentin probably provides the nucleus with mechanical support. Tubulin is the major protein of the microtubules.

2.2.2.7 Muscle fibre types

On the basis of colour, on macroscopical examination, muscle fibres were classified as red and white fibres as early as 1678 by Stefano Lorenzini (cited in Dubowitz, 1985). The red fibres are smaller fibres found in postural muscles like the back and neck muscles. They were supposed to be slow twitch and weaker muscle fibres, but could act for prolonged periods without tiring. On the other hand white fibres are large and powerful, but tire quickly like the muscle of the limbs used for carrying, walking and jumping.

Modern histochemistry, enabling the identification of enzyme systems and other cell constituents, now made possible the correlation of the functional activity of individual fibres with their morphology. The standard ATPase reaction test at pH 9,4 initially led to the adoption of the two fibre system, with the type 1 fibres being weak and the type 2 fibres strong in reaction. The adoption of the 2 fibre system was practical and useful for the assessment of both normal and diseased muscle. Later, type 2 fibres were further classified into subgroups as 2A, 2B & 2C on the basis of the standard ATPase reaction at pH 4,3 or 4,6. This further classification was useful in demonstrating selective physiological and morphological changes in muscle. It helped explain the process of development and maturation of fetal muscle.

Ultrastructural correlation with histochemical and physiological features of muscle were much easier in the animal models because examination of muscles, composed of a single type of fibre without mixing, was possible. In the human this was made difficult as the human muscles were of a mixed type and was easier to relate the predominance of a fibre type. The many histochemical techniques to identify the physiological characteristics of muscle were well documented (Dubowitz, 1985).

2.2.2.8 Blood vessels and nerve supply

The vascular and nerve supply to the myofibres is conveyed in the endomysial tissue. Type 1 fibres have more capillaries than type 2. The nerve fibres from the perimysium enter the endomysium to terminate on individual myofibres at points known as neuromuscular junctions.

2.2.2.9 Muscle spindles

Specialised striated muscle fibres (4-16) enclosed within a sheath or capsule forming muscle spindles are located in the perimysial tissue adjacent to blood vessels and nerves. These specialised fibres are of two types, one being called a nuclear bag fibre, having a collection of nuclei in the central area of the fibre, and the other, the thinner chain fibre with chains of nuclei for much of the length of the fibre. The fibres of the spindle known as intrafusal fibres are supplied by their own motor and sensory nerve fibres. The muscle spindle, a proprioceptive sensory organ, is a comparator of static and dynamic stretch occurring in the skeletal muscle and is also responsible for the maintenance of muscle tone.

2.2.2.10 Satellite cells

Rarely occurring in mature adult muscle are satellite cells which were first identified by Mauro (1961). Satellite cells, presumed to be embryonic remnants of myogenesis, are characteristically located between the basement membrane and the plasmalemma of the muscle fibre. These cells are thought to be reserve cells which become activated in the muscle in times of stress, proliferate by mitotic division, fuse to form myotubes and finally form new myofibres (Campion, 1984). Research findings on satellite cells after their discovery by Mauro (1961) are discussed in greater detail later on in this chapter.

2.3 MUSCLE REGENERATION STUDY TECHNIQUES

According to Lipton (1979) regenerating cells were commonly found in biopsies from young dystrophic patients, but the frequency of such cells declined steadily in patients older than 5 years. However, there was some degree of regeneration in advanced cases of up to 10 years or longer after the onset of clinical symptoms. It is well documented in structural studies (Lipton, 1979) that regenerative activity in DMD progressively declined, and that formation of functional myofibres after the onset of clinical symptoms was not possible. In the older dystrophic patients, 50 to 90% of the muscle fibres were replaced by connective tissue. In the young DMD patients, normal, atrophic, hypertrophic and regenerating fibres were a common feature (Lipton, 1979).

To identify the mechanisms involved and leading up to the muscle regeneration defects above, many techniques were tried and tested to induce regeneration in living animals and under culture conditions in the laboratory. Speidel as early as 1938 used living frog tadpoles to study regeneration. He provided morphological evidence for the progressive changes which took place from mononucleated spindle shaped myoblast up to the formation of myofibres. These findings held true for the animal and human muscle regeneration in subsequent studies that followed and were similar to those obtained in the current study (Figs. **1** & 2). Some of the techniques that are now obsolete and those that are currently used in regeneration studies are discussed.

2.3.1 Animal models used in regeneration studies

A variety of animal models from the animal kingdom were used in skeletal muscle regeneration research. Some of these included :- insects like the blowfly (Gregory et al., 1968); anuran species and tadpoles (Hsu, 1974; Trupin, 1976; Mazanet et al., 1982; Takahama, 1983); amphibia and reptiles (Thornton, 1938; Lentz, 1969; Carlson, 1979, Hay 1959/1979); birds - chick (Bischoff and Holtzer, 1969; Wright, 1985;) and quails (Konigsberg et al., 1975; Lipton, 1977a&b); fish (Sandset and Korneliussen, 1978); rats (Mong, 1977; Kelly, 1978a&b) and mice (Shafiq and Gorycki, 1965; hamsters (Tautu and Jasmin, 1982; Karpati et al., 1983; Jasmin and Bokdawala, 1970); guinea pigs (Hess and Rosner, 1970); rabbits (Yarom et al., 1976); foetal pigs (Campion et al., 1978); monkeys (Allbrook et al., 1966); fruit bats (Church et al., 1966; Church, 1969); cats and dogs (Ishikawa, 1966) and human muscle (Yasin et al., 1977; Thompson et al., 1981; Minguetti and Mair, 1981; Delaporte et al., 1984; Jasmin et al., 1984a&b).

2.3.2 Grafted muscle and implants

Some of the studies following the development of muscle (Ishikawa, 1966; Shafiq et al., 1967) used fixed and stained muscle preparations for microscopical study taken directly from foetuses, young and adult animals and from humans. Other studies involved the grafting or interchanging of muscle segments between animals (Jasmin and Bokdawala, 1970; Schmalbruch, 1977), and autografting of muscle mince (Snow, 1979).

Jasmin and Bokdawala (1970) reported success with normal hamster littermates, but the grafts became scar tissue when placed into dystrophic hamster. Failure in such grafts were attributed to the pathogenicity of the disease. Muscle grafts were also performed on the same animal with muscle being transferred from one limb to the other (Schmalbruch, 1977). The graft technique of study was over protracted periods and, even though the muscle grafts were placed within the animals they suffered poor innervation. Mong (1977) used rats, mincing their muscle and leaving them inside the animals as implants. In some of the rats, the muscle carrying the regenerates were denervated. He suggested that innervation was important for the late phase in regeneration and for the differentiation of the fibre types.

Regenerative and degenerative responses in minced and autografted muscle were also investigated (Snow, 1977a). Snow (1977b/1978/1979) performed autoradiographic studies on minced radioactive hind limb muscles of young rats. [^{3}H]thymidine was injected into young rats to achieve labelling of satellite cells. Snow then transplanted the muscle to untreated littermates. New regenerated myotubes with labelled nuclei confirmed that satellite cells in young rat muscle were capable of differentiating into myotubes after muscle injury. Other investigations on muscle grafts included free muscle grafts (Carlson et al., 1979), grafts between normal animals and dystrophic animals (Cosmos et al., 1979) and autogenous muscle transplants (Hall-Craggs, 1979). Labelled satellite cells obtained from clonal cultures were also implanted into the original donors, namely rats and quails (Lipton and Schultz, 1979).

2.3.3 Denervation and muscle injury

Apart from minced muscle implants, animal muscle injured, ***in situ***, in various ways, was also used in muscle regeneration research. In some cases the nerve supply to the muscle under investigation was removed to study the influence of denervation of muscle during the regeneration process (Mong, 1977; Kelly, 1978b). Kelly (1978b) denervated the soleus and extensor digitorum longus muscle of developing and mature rats to study growth patterns in the two different types of muscle using [^{3}H]thymidine labelling. Campion et al., (1978) destroyed the cervical spinal cord of fetal pigs by cauterization at 45 days of gestation to investigate muscle development. Crush injury and [^{3}H]thymidine labelling of mdx mice muscle were also employed (Grounds and McGeachie, 1992).

During muscle regeneration in rats and rabbits, after injury by ischaemia, freezing or autografting, the early stages of presumptive myoblast proliferation, myoblast fusion and the development of multinucleated myotubes occurred within the basal laminar tube. The basal laminar tube (Vracko and Benditt, 1972; Schmalbruch, 1976) was said to provide a scaffolding for orderly cell replacement. Regeneration of muscle was also shown to take place almost exclusively within the sarcolemmal tubes obtained from the surviving basement membrane of dry ice injured muscle of 2 week and 1 year old chicken pectoral muscles. Focal continuities between satellite cells and old myofibre were observed, and these focal continuities were regarded as possible evidence of fusion between satellite cells and the necrotic fibre. Activated satellite cells characteristically demonstrated numerous cell processes, which made them resemble macrophages (Nichols and Shafiq, 1979).

In one study, the frog muscle was injured by pinching through the skin, and other muscles of the frog was crushed, ***in vitro,*** and examined at different intervals for the effects of such injury (Mazanet et al., 1982). Colchicine effects on regeneration of transected tibialis muscle of mice (Pietsch, 1961), cold effects on rat muscle (Price et al., 1964), the use of hot brass tips inserted into the muscle (Shafiq and Gorycki, 1965), damage to soft tissue and fracture to limbs of anaesthetized monkeys with a strike force (Allbrook et al., 1966), freezing and ischaemia (Vracko and Benditt, 1972), segmental squeezing of the soleus muscle with a surgical clamp and use of hot Ringer's solution in rats (Schmalbruch, 1976), denervation and tenotomy (McGeachie and Allbrook, 1978), and micropuncture injury with a tungsten wire causing necrosis to all fibres pierced (Carpenter, 1990) were some of the methods of injury used in muscle regeneration research.

Studies on epimorphic regeneration exhibited by amphibia and reptiles were conducted on amputated limbs of these animals (Thornton, 1938; Carlson, 1979; Hay 1979).

Yarom et al. (1976) injected soluble gold and inflicted extreme cold injury to the extraoccular muscles of rabbits. Myoblasts and myotubes were shown capable of taking up the gold tracer, thus giving credibility to the opinion that myoblasts and myotubes performed as phagocytes during the stages of muscle regeneration.

2.3.4 *In vitro* cultured muscle

Geiger and Garvin (1957) were the first to culture human muscle to identify inherent peculiarities of muscle cells of progressive muscular dystrophy

patients. It was felt that if muscular dystrophy was due to changes intrinsic to the muscle fibre, these pathological changes ought to be identified in muscle cultures (Witkowski and Dubowitz, 1975; Witkowski, 1977).

In recent times, muscle culture techniques in a controlled laboratory environment were the choice in the study of muscle regeneration. The techniques used either enzymatically dissociated mononucleated muscle cells or minced explants.

The culture media, even though varying slightly in different laboratories, usually comprised of base medium containing synthetic ingredients in optimal concentrations to promote growth. To the base medium, made up of synthetic organic and inorganic nutrients in physiological buffers, was added foetal bovine serum, chicken embryo extracts and antibiotic/ antimycotic solutions (Yasin et al., 1977; Jasmin et al., 1984a). Foetal bovine serum was replaced by horse serum when fusion of cells was the requirement (Hauschka, 1974).

Askanas (1979) considered regeneration of diseased human muscle in cultures a valuable tool to study the pathogenesis of neuromuscular diseases, and that it presented a new frontier in the study of neuromuscular disorders. According to Askanas the aim in using cultured diseased human muscle was to attempt to reincarnate in cultures the structural changes and biochemical defects that were observed in biopsied muscle. Furthermore, it was felt that cultures might reveal unknown morphological and biochemical defects and enable attempts at treatment of the identified abnormalities, ***in vitro***.

2.3.4.1 Myofibres and muscle explants

In earlier research, explants prepared from muscle biopsies obtained from normal and dystrophic patients were cultured in plasma clots using Carrel flasks. Tissue culture medium called TC.199 containing human AB serum was used as the feeding solution (Geiger and Garvin, 1957; Skeate et al., 1969; Bishop et al., 1971; Bischoff, 1972; Dubowitz, 1973). Mendell et al. (1972) reported shrunken and pyknotic nuclei within the first two days after incubation of human muscle explants cultured in Maximow double coverslip chambers on collagen-coated plastic coverslips. These explants were fed 1 drop of growth medium 3 times a week. In this study an attempt was made to investigate the ultrastructural characteristics of human muscle in culture.

The above methods were beset with problems of necrosis, invading cells and perfusion or proper nutrient supply of the explants under investigations. Migration of sprouting cells away from the explant in a clot was restricted. In addition, the preparation of muscle cell line clones were not possible with this method (Yasin et al., 1977).

The muscle explant technique, nowadays, has come a long way since the early clot culture technique. Minced muscle grown as free floating explants in aqueous unclotted culture media provided better migration and growth of presumptive myoblasts (Askanas and Engel, 1975; Tautu and Jasmin, 1982; Delaporte et al., 1984; Jasmin et al., 1984a). Some of the disadvantages of the explant technique were that it encouraged prolific growth of fibroblasts together with other connective tissue cells which became a nuisance as the cultures got older to the extent that the fibroblasts outgrew and restricted the growth of myoblastic cells. To overcome this, Askanas

and Engel (1975) cultured explants for two weeks, and then transferred the explants to a new flask thus achieving less contamination by fibroblast growth.

Jasmin et al. (1984a) advocated gentle agitation of the culture flasks every day to overcome the adherence of the explants to the culture flask coated with gelatin. Ecob-Prince and Brown (1988) cultured human muscle explants together with mouse spinal cord. The growth medium used in this study included 25% human placental cord serum, 10% chick embryo extract and 6% glucose.

Single myofibres from rats (Bischoff, 1972/1975/1979) and from Japanese quail (Konigsberg et al., 1975; Konigsberg, 1979) were also cultured to investigate myoblastic response. Single whole fibre cultures which were performed from teased muscle seemed an inefficient way of producing mononucleated cells. Rubin et al. (1979) cultured single fibres released from rat muscle by collagenase digestion. These fibres maintained their striations and young myotubes were seen only in the vicinity of adult degenerate myofibres.

Culture media and the explant method of muscle culture in use at the present time is discussed in greater detail under methods and material in the following chapter.

2.3.4.2 Dissociated mononucleated myoblasts

The dissociation technique of muscle cultures, using single mononucleated cells isolated by proteolytic enzyme digestion of muscle fragments, was born

and gained impetus with the discovery of the satellite cell by Mauro (1961). Proteolytic enzymes namely, trypsin, pronase, collagenase, papain and ficin were tried for their ability to free mononucleated cells from rat muscle (Bischoff, 1974). The school of researchers (Yasin et al., 1977; Thompson, 1980) advocating the use of the dissociation technique of muscle culture, were of the opinion that this technique was better than the explant technique which, they felt, had many limitations. Some of the limitations enumerated were:- that the explants were relatively opaque such that the occluded myofibres in the centre of the explants could not be examined; that cell survival could not be quantitated; that the metabolic environment of the central fibres was different from those on the surface and that cells on the surface would be the first to suffer any toxic elements present in the media.

Cossu et al. (1980) isolated mononucleated cells from normal and dystrophic mice as well as from dystrophic human muscle using collagenase and hyaluronidase together with trypsin. Most dissociation methods found the use of trypsin alone or the combination of trypsin and collagenase efficient enough to liberate mononucleate cells containing presumptive myoblasts (Hauschka, 1974; Yasin et al., 1977; Thompson, 1980; Ecob-Johnston and Brown, 1981; Delaporte et al., 1990).

Both fresh muscle and muscle stored for up to 96 h at 4°C were cultured successfully (Yasin et al., 1977). The dissociation method of culture essentially required the enzymatic digestion of small pieces of muscle which were first cleaned by the removal of visible connective and adipose tissues. The teased muscle was incubated for 15 minutes while being agitated in a waterbath at 37°C. A trypsin-collagenase enzyme mixture was the usual

choice at the Institute of Neurology and Neurochemistry, London, where I was taught the technique by Rose Yasin. After 15 minutes the supernatant was treated with an enzyme neutralising serum-rich medium. The cell suspension obtained was centrifuged and the pellet formed was resuspended in growth medium. Pellets were obtained by further digestion of the remaining muscle for at least three sessions. The pellets resuspended in growth medium were pooled at the end and filtered through a fine mesh to discard muscle debris. Approximately 200 cells per 25 cm^3 glass Petri dishes, precoated with gelatin, was incubated at 37°C in a humidified atmosphere containing 5% CO_2. The number of cells that adhered to the flask the next day gave an indication of the plating efficiency of the technique. The dissociation technique was laborious and time consuming. Furthermore, a mixture of fibroblasts and myoblasts grew in dissociated cell cultures, and it was not possible to differentiate between them (Yasin et al., 1977). Cluster formation was often observed in these cultures (Walsh et al., 1981; Thompson et al., 1983). There was also a greater chance of contamination of cultures with this technique. The same end result of obtaining single cells could be achieved using the explant technique which is less time consuming, a simple technique, easier to handle and with less chances of contamination.

2.4 REVELATIONS IN MUSCLE REGENERATION STUDIES

Records showed that muscle regeneration studies began over 135 years ago (Bottcher, 1858; Waldeyer, 1865 - cited in Campion, 1984). Muscle regeneration studies may be grouped into two major experimental situations. The first group entailed the study of regeneration by microscopical comparisons of muscle from foetuses, in the young and in adult human, and

many animal species mentioned in previous sections of this chapter with some references. Muscle autografts, muscle transplants, minced muscle implants, denervated muscle and muscle injured in a variety of ways were employed to pursue the development and regeneration of muscle.

The other group employed tissue culture techniques. Some of the findings, using the early clot culture technique, revealed that normal and diseased DMD muscle were capable of forming striations in culture similar to normal muscle. Explant cultures performed in this way together with foetal spinal cord implants of animals suggested a neurogenic cause to the pathogenesis of muscular dystrophy (Dubowitz, 1973).

The explant and the dissociation techniques in current use, were used successfully to grow primary cells and promote fusion of myoblasts in cultures leading to the development of multinucleated myotubes. The myotubes continued development leading to young striated muscle fibres which were observed to undergo contraction in cultures, thus proving the efficacy of the culture techniques to promote differentiation of muscle cells, ***in vitro***. This method of culture, unlike the clot method, did not restrict, but promoted the free migration and proliferation of mononucleated cells. Preparation of muscle cell clones were possible with either method.

On the other hand, Konigsberg (1963) expressed caution in the analysis of results from culture which might be completely an atypical response, unrelated to the processes occurring, ***in vivo***. Identification of ubiquitous tissue cells were difficult in cell cultures. Also the progeny of differentiated tissue may undergo modulation in response to exposure to the artificial environment. Konigsberg went on to state that it was also difficult to

assess cellular damage by enzymes and other mechanical manipulations resorted to in the preparation of cultures.

In general it may be summarised that the growth characteristics of diseased and normal muscle did not indicate any appreciable growth and morphological differences in culture (Dubowitz, 1973).

All earlier work, irrespective of the investigative technique used, had one thing in common, and that was to test the various hypotheses that existed on the pathogenesis of muscular dystrophy. These being mentioned earlier were:- a defect in cell membranes, a primary lesion of the motor neurones, a lesion in the muscle microcirculation and an anomaly of the connective tissue, with most of the evidence favouring the first possibility (Yasin et al., 1979; Cullen and Jaros, 1988).

In investigating a number of these hypotheses, the mdx mouse was and is still used widely in DMD research. However, the mdx mouse which served as an animal model posed a difficulty in that the muscle of the mouse had the ability to regenerate after initial degeneration, but this was not the case with human DMD. This difference was attributed to plasma membrane lesions in the human muscle which were not seen in the mdx mouse (Cullen and Jaros, 1988). The membrane theory or the basic abnormality in the plasma membrane of DMD muscle fibres (Mokri and Engel, 1975) was further supported by the elevated creatine kinase enzymes found in the serum of Duchenne dystrophic patients together with elevated intracellular calcium levels (Dubowitz, 1989a). Changes in intramembranous particles, revealed in freeze fractures as well as focal lesions (Mokri and Engel, 1975) lent further support for the membrane defect in DMD patients.

With the identification of the gene defect and the gene product, dystrophin, many of the conflicting findings as cluster formation in cultures of DMD and a neurogenic or ischaemic cause of the defect in muscular dystrophy have become redundant and hold only historic and academic merit.

Nevertheless, the culture techniques used are not without merit and will be in continuous use in the future. In recent times the techniques have been perfected to isolate and harvest massive numbers of muscle cell clones (Konigsberg, 1963; Hauschka, 1974; Yasin et al., 1982). The proposed myoblast transplant and gene transfer therapies together with the identification of the different dystrophic types, including proper prenatal diagnosis and carrier status in patients will make use of these cultured cells for many years to come.

On account of the vast amount of literature available on muscle development and regeneration, subsequent review on the findings in a number of muscle regeneration studies will be compartmentalized into specific subheadings to facilitate collating the information.

2.4.1 Origin of myoblasts during muscle regeneration

The origin of myoblasts is still a debatable issue. The origin of the myogenic cell has not been proven beyond doubt in any of the muscle regeneration models used (Carlson, 1973; Sloper and Partridge, 1980). There are two schools of thought on this issue. One school supports the hypothesis that satellite cells are the precursor cells involved in muscle regeneration and this is the current consensus (Church et al., 1966; Moss and Leblond, 1970/1971; Ontell, 1975; Schmalbruch, 1976; Snow, 1977a&b/1979; Bischoff, 1979). The other school believes that myonuclei in muscle were

also capable of undergoing dedifferentiation to form new cells if and when muscle regeneration was required (Hay, 1959/1979; Lee, 1965; Reznik, 1969/1976; Hess and Rosner, 1970; Teravainen, 1970). There were suggestions that connective tissue cells such as fibroblasts and macrophages may also be involved in the contribution of new cells during regeneration (Sloper and Partridge, 1980).

2.4.1.1 Satellite cells as presumptive myoblasts

2.4.1.1.1 Satellite cells

Satellite cells named by Mauro (1961) were first identified in the tibialis anticus muscle of the frog, using an electron microscope. According to Mauro, the cells characteristically had little cytoplasm and lay between the basement membrane and the plasmalemma of the myofibre. The plasmalemma of the satellite cell was distinctly separate from the basement membrane on the outer region and the plasmalemma of the myofibre on the inner surface. The plasmalemma of the myofibre was depressed at the position occupied by the satellite cell, pushing the myofibrils inward so that the contour of the basement membrane did not show alteration. The nuclei of these cells had electron dense peripheral heterochromatin, and due to the paucity of cytoplasm they could easily be mistaken for myonuclei. Identification of the satellite cell was virtually impossible with the light microscope.

Mauro (1961) speculated three possible mechanisms of their derivation in the peripheral region of the myofibre. The first one was that surviving myonuclei in the degenerating muscle fibre gave rise to single cells by

"gathering up" cytoplasm from the myofibre by the dedifferentiation method (Hay, 1959), and that in the normal state, there was a slow rate of production of these cells. In the event of any muscle stress, the satellite cell production was increased. Secondly, a more acceptable hypothesis by most biologists was that these cells were embryonic remnants of early myogenesis. During the course of fusion of myoblastic cells to form myotubes and finally myofibres, some cells remained in the sublaminar position as dormant cells which had the potential to recapitulate when muscle damage occurred. Mauro also suggested that both mechanisms may be operating as the source of satellite cells. "Wandering" cells, penetrating the basement membrane and lying in the sublaminar position until called into activity was the last possibility.

A volley of research followed on this satellite cell path. Enough evidence was presented to demonstrate that satellite cells were independent of the adjacent myofibres (satellite cells review - Campion 1984). Freeze-fracture studies did not reveal any membrane specializations between satellite cells and myofibre plasmalemma (Schmalbruch, 1978). Some workers suggested that there was a uniform distribution of satellite cells along the length of the myofibre (Campion, 1978; Schultz, 1978; Snow, 1981). Others reported increased numbers of satellite cells near myoneural junctions (Kelly, 1978b; Cardasis, 1979). Satellite cells were present also in the intrafusal fibres of the muscle spindle (Snow, 1977a). Apparently, satellite cell population was closely tied to that of the myonuclei within the myofibre (Kelly, 1979) and satellite cells were considered to behave as stem cells (Moore, 1979).

Despite increased satellite cells in denervated muscle, ultimate failure in regeneration was attributed to the possible inability in satellite cell

production to outrun degeneration, to cytotoxic effect produced by degenerating fibres and to the absence of the trophic effects of the nerve (Ontell, 1975). The ability of satellite cells from dystrophic human and murine muscles to differentiate in cultures was not found to be different from that of normal muscles (Cossu et al., 1980) even though others reported differently, example, cluster formation in diseased muscle cells in culture (Yasin et al., 1979; Thompson et al., 1981).

Carlson (1979) enumerated a number of points on the characteristics of muscle regeneration at that time. Some of these included:- that satellite cells were probably the major source of myoblasts; that phagocytosis played a major role in the removal of damaged muscle; that most regeneration occurred within the confines of the basal laminar tube; that nerves were not required for the early differentiation and morphogenesis, but only for functional differentiation and maintenance; that there was a fairly direct relationship between the amount of damaged and regenerating muscle; that the morphology of the regenerate was usually imperfect and that the gross morphogenesis, internal architecture and quality of the regenerate depended on physical factors and the functional environment. According to Carlson (1979), the progression from myoblast to mature myofibre at the cellular level was same as that described for the embryonic development of muscle. At the supracellular level, the reorganization of an isolated regenerating muscle was different from that in the embryo.

2.4.1.1.2 Morphology of satellite cells

The morphology of satellite cells observed since first described by Mauro (1961) varied as most sublaminar cells observed in subsequent studies,

irrespective of their morphology, were labelled as satellite cells. Satellite cells of young animals were shown to have abundant cytoplasm rich in organelles and that organelles were increased in both quality and quantity (Schultz, 1976). The morphology of satellite cells was said to change during mobilization of these cells for regenerative purposes (Mazanet et al., 1982). Sublaminar cells which were fusiform, having short or long cytoplasmic tails originating from either end of the bulging cell body, with the tails extending for considerable distances below the sarcolemma were also considered as satellite cells (Mazanet et al., 1982). Some of the satellite cells were described as having lateral cytoplasmic projections residing in grooves over the surface of myofibres, and these were observed in freeze-fracture studies (Schmalbruch, 1978).

The physiological state of the satellite cells whether being active or inactive depended on the organelle and the fine structural picture of its cytoplasm (Campion, 1984). In the frog sartorius muscle the organelle content of different satellite cells were variable such that the morphology of satellite cells were not in keeping with that first described by Mauro in 1961 (Franzini-Armstrong, 1979).

2.4.1.1.3 Satellite cell distribution and numbers

The proportion of satellite cells of muscle nuclei was 4% in the anterior tibialis and 8% in both the soleus and the diaphragm of adult rats giving satellite numbers of 900, 4900 and 5300 per mm^3 muscle (Schmalbruch and Hellhammer (1977). On the other hand, according to Allbrook (1981) the satellite cell count in normal muscle varied between 1 to 5%. However, according to Campion (1984) the physiological state of the muscle was

reflected in the state of activity of the satellite cells with increased numbers being present in young and growing animals as compared with the old in which case satellite cells showed a decline with an increase in age. Satellite cells were seen in the muscle of a 73 yr old man (0,6%) with human adult average being 4% (Schmalbruch and Hellhammer, 1976).

The differences, if any, between the postnatal satellite cell and the sublaminar mononucleated cell of embryonic origin in adult were not known (Campion, 1984). The differences in distribution of satellite cells in different muscles in the same animal, as shown for the soleus and the extensor digitorum longus muscles of the rat, was attributed to a distinction laid down in early myogenesis (Kelly 1978b). Frog sartorius muscle showed the highest content of satellite cells of all other muscles studied, the figure being 12-13% (Franzini-Armstrong, 1979). Wakayama and Schotland (1979) reported that the greatest numerical increase in satellite cells occurred in DMD patients when the clinical manifestations worsened rapidly.

Satellite cells were not observed in pairs, but it was not uncommon to find satellite cells near myonuclei (Ontell, 1974). Satellite cells were reported to be found more frequently in red fibres than in white fibres of tadpole muscle (Takahama, 1983).

Satellite cells were found in skeletal muscle of most species of vertebrates. Even though satellite cells were reported in cardiac muscle of decapods they were not observed in cardiac muscle of vertebrates (Midsukami, 1981). According to Midsukami, cardiac satellite cells in the crustaceans usually occurred in the area overlying the intercalated discs.

2.4.1.1.4 Satellite cell migration

Migration of satellites from damaged to undamaged areas of muscle was also suggested (Mastaglia and Walton, 1971; Schultz et al., 1985; Hughes and Blau, 1990). Migration of myoblasts in injured muscle was thought to be due to chemotactic influence exerted by the injured myofibre (Watt et al., 1994). However, poor migration of injected donor myoblasts was said to be the cause of poor positive dystrophin response in myofibres of recipients (Satoh et al., 1993). This somewhat negates the migration story of myoblasts.

2.4.1.1.5 Satellite cells as myogenic precursors of regeneration in dissociated and single myofibre studies

The source of myogenic cells during skeletal muscle regeneration was attributed to satellite cells by many studies on muscle regeneration (Campion, 1984). Satellite cells supposedly behaved as stem cells when the adequate stimulus to promote muscle regeneration was made available (Moore, 1979).

Bischoff (1979) performed a myogenic cell suspension study with muscle treated with enzymes that digested the basal lamina and with those that did not. Cells, isolated by digestion of the basal lamina with trypsin, when cultured formed myotubes and cross striated myofibres. Those enzymes that did not digest the basal lamina released non-myogenic cells which in cultures produced fibroblasts, fat cells and macrophages. The deduction from this experiment was that the myogenic cells released from the sublaminar position were myogenic stem cells or satellites.

In experiments with single fibres cultured in a fibrin clot, Bischoff (1979) reported that clot retraction of the fibres left clear zones between the clots. Cells with myogenic potential proliferated within these clear zones, and Bischoff proposed that these myogenic cells were derived from satellite cells. Furthermore, electron micrographs of single fibres from clot cultures, taken at zero time in culture, revealed gross degenerative features. The interpretation/differentiation between interstitial and satellite cell as well as between the basal membrane and plasmalemma presented in that work was debatable. The cell referred to as satellite cell at zero time, in one instance appears to be an active macrophage. Furthermore, in some of the pictures, the plasmalemma is more intact and conspicuous than the basement membrane, while the opposite is more often the case in degenerate fibres. It would have been interesting had he studied the clot itself with the EM to observe if there were any myonuclei trapped within the clots inside the myofibre.

According to Konigsberg et al., (1975) the solution to both the hypotheses on satellite cell versus the myonuclear derivation of precursor cells of regeneration was complicated by two difficulties. The one was that muscles of animals experimentally injured were observed at progressively longer intervals after the time of injury. The other was that, the identification of both the fragments budding and the definitive identification of satellite cells, required resolution afforded by the EM.

Konigsberg et al. attempted to circumvent the first difficulty by continual monitoring of teased single muscle fibres of quail cultured with a phase contrast photomicroscope. All myonuclei from the cultured myofibres were reported to have disappeared. Furthermore, from 505 fibres cultured, only

110 gave rise to some cells and only two colonies were formed. At the outset of the cultures, many of the surviving single cells were reported having a tangential orientation rather than parallel to the fibre axis. From the results obtained, Konigsberg et al. suggested satellite cells as the precursor cells although the possibility of budding was not entirely excluded. The culture technique of single fibres, in present day terms, was obviously inefficient as can be interpreted from some of the results obtained in the above study.

2.4.1.1.6 Satellite cells as myogenic precursors of regeneration in labelled muscle in *in vivo* and explant studies

Moss and Leblond (1970/1971) using radioactive thymidine labelling in young rats concluded that satellite cells contributed to the increase in the number of nuclei during muscle regeneration. The results obtained in their study are shown in the tables **a** and **b** below.

Table a: Nuclear labelling in tibialis anterior muscle of 20-30g male rats at various times after a single injection of thymidine-^{3}H as seen in electron microscope autoradiographs (Moss and Leblond, 1970).

Time elapsing between thymidine-^{3}H injection and sacrifice - *hr*	Number of nuclei labelled	
	Satellite cell nuclei	True muscle nuclei
1	20	0
6	11	0
10	24	0
24	8	2
48	12	11
72	4	24

Table b: Labelled satellite cell nuclei and true muscle nuclei as a percentage of the total labelled nuclei in the tibialis anterior muscle of 30g rats at various intervals after a single injection of ^{3}H-thymidine (Moss and Leblond, 1971).

Time elapsing between ^{3}H-thymidine injection and sacrifice - *h*	Total labelled nuclei classified (5 rats at each time)	Percent of labelled nuclei		
		Satellite cell nuclei	True muscle nuclei	Standard error
1	321	100,0	0	1
18	616	95,1	4,9	0,8
24	377	84,9	15,1	3,0
48	559	51,0	49,0	1,4
72	296	34,8	65,2	2,4

Between 1 and 10 h after injection, 100% of the labelled nuclei belonged to satellite cells. The satellite number of those animals were given as 10 to 15% of the total myofibre nuclei (Moss and Leblond, 1970).

According to Moss and Leblond (1970), only 3% of the nuclei under the basement membrane were labelled 1 h after injection of the labels and that they belonged to satellites. In the second instance (Moss and Leblond, 1971) the table shows a larger number of satellites was labelled at 18 h compared to 1h after injection of the labels, but dropped drastically at 24 h. The number of satellites cells labelled after 24 h were conflicting in the two tables above. Even if one considers the doubling time of satellite cells, the labelling of satellite cells and myonuclei in figures and not as a percentage are quite puzzling. The number of myonuclei taking up the labels do not add up! After 48 hours, satellite cell counts comprised 51% and myonuclei 49% of the labelled nuclei (Moss and Leblond, 1971). The labelled

myonuclei were regarded as nuclei belonging to satellite cells that had already fused with the myofibre. However the study did not attempt to prove the derivation of the satellite cells.

Muscle from neonatal rats were labelled with [^{3}H]thymidine and explants were cultured to identify the role of satellite cells during myogenesis (Hsu et al., 1979). Unfortunately, the explants were not examined to identify the derivation of new cells. Instead labelling was inspected in the myotubes that formed from the young cells that sprouted from the explants. The labelling protocol rested on the labelled neonatal satellite cells fusing with adjacent myofibers to produce increased number of myonuclei. Only satellite cells had taken up labels 10 h after injection. By 24 h, 15% of the myonuclei were labelled. Cultures of explants, taken 8 h after injection of labels, produced myotubes from the young cells that spread on the culture surface. These myotubes showed labelled satellite cells and myonuclei. Labelled myonuclei prevailed in explants taken between 4 to 6 days, and satellite cells revealed a dilution of the labels at this time. Exact figures of counts were not given in the study. Moreover, because the animals were still very young and in the growth phase, no useful interpretation can be made from this study. However, the conclusion in this work was that satellite cells were the myogenic elements in their, ***in vitro,*** *study* (Hsu et al., 1979).

In another study, the soleus and extensor digitorum longus muscles with compensatory hypertrophy which showed no apparent ultrastructural damage revealed satellite cells to have taken up [^{3}H]thymidine labels. This implied that injured or regenerating muscle were not the only muscle that were capable of forming new fibres (Schiaffino et al., 1979).

2.4.1.1.7 Satellite cells as myogenic precursors in labelled muscle autografts and transplants.

Snow (1978/1979) attempted differential labelling of myonuclei and satellite cell nuclei with three experimental groups of young rats to gain clarity on the origin of myoblasts during regeneration. The results of the study (Snow, 1979) are shown in the tables below.

Summary of control and experimental quantitative data for myonuclear labelling in series I and satellite cell labelling in series II (Snow, 1979).

Nuclear classification Series 1 (5-6 wk old rats)		Total nuclei observed	Labelled nuclei observed	Percentage of labelled nuclei	Percentage of mononucleated cells beneath the external lamina
Control muscle	Myonuclei	1 191	246	20	
	Satellite cell nuclei	156	none	0	11,6
Regenerating muscle	Pyknotic nuclei	1 018	202	19,6	
	Viable nuclei	69	none	0	6,3

Nuclear classification Series 2 (15-17 wk old rats)		Total nuclei observed	Labelled nuclei observed	Percentage of labelled nuclei	Percentage of mononucleated cells beneath the external lamina
Control muscle	Myonuclei	2 061	none	0	
	Satellite cell nuclei	377	86	22,8	15,4
Regenerating muscle	Pycnotic nuclei	1 528	22	1,4	
	Viable nuclei	117	27	23,1	7,1

In the first group, [^{3}H]thymidine was initially injected into pregnant rats with subsequent injections administered to the new born to effect labelling of myonuclei that would have occurred during the fusion and maturation of the fibres in the young. After 5-6 weeks maturation, muscle was injured by mincing and autografted. After 8-24 hours, inspection of thick and thin sections of the regenerates revealed labels in 20% of the pyknotic nuclei but not in the viable mononucleated cells which made up 6% of the total sublaminar nuclei. The same percentage of myonuclei were labelled in the uninjured controls. It is questionable that no satellite cells were labelled even if one had to take the dilution factor into account, especially if one considered the fact that satellite cells were considered reserve myoblasts. Furthermore, the cause of death of all the myonuclei in the autografts also need an explanation.

In the second group, labelling of satellite cells was effected in uninjured muscle of 15-17 day old rats which had relatively high counts of satellite cells of about 15%. One hour after a high pulse dose, muscle was minced and autografted as before. Results in regenerates, 8-24 hour after injury, showed 23% of sublaminar mononucleated cells labelled, tallying with the satellite cell counts before injury. Again a question arises as to why only 23% of satellite cells took up the labels before injury if these were young animals with active satellite cells. In this group, a number of pyknotic nuclei were also labelled and the reason for this was not adequately explained.

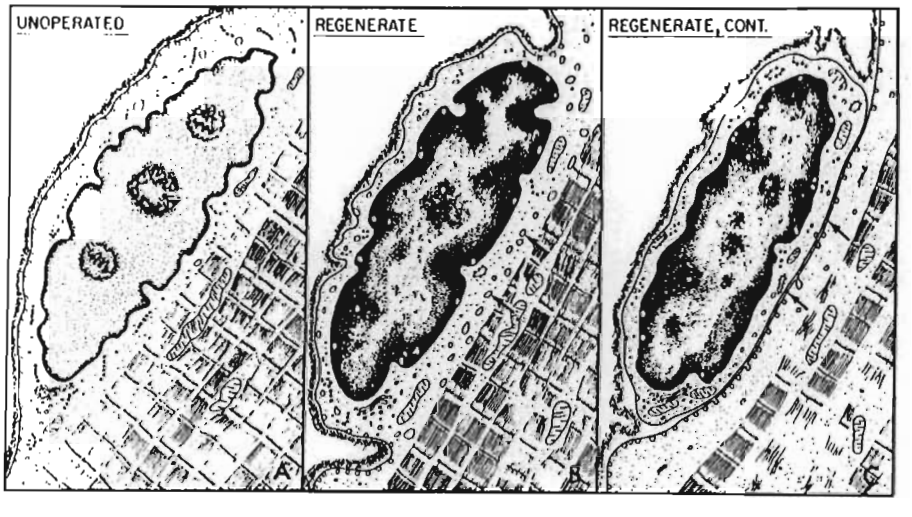
The third group of rats was used to test the survival and differentiation of the satellite cells. Labelled muscle mince was implanted into nonradiated littermates and examined 4-6 days later. Approximately 7% of the nuclei of the myotubes were labelled even though control muscle had a count of 31%

labelled satellite cells. The dilution of labels during mitotic division as well as the possible death of some of the satellite cells was forwarded as the reason for the discrepancy in the labelling indices. Snow (1979) concluded that satellite cells were capable of surviving injury to promote regeneration, but that the role of satellite cells in adult muscle remained uncertain.

2.4.1.2 Myonuclear derivation of presumptive myoblasts

2.4.1.2.1 Dedifferentiation of myonuclei

The dedifferentiation of myonuclei segregating some cytoplasm around it and budding of new cells was thought to be similar to the mechanism of regeneration demonstrated in some amphibians and reptiles after amputation of their limbs (Thornton, 1938; Carlson, 1979; Hay, 1959/1979). According to Hay (1979), after amputation of the newt limb, the distal epithelium covered the wound. A blastema then formed from cells which dissociated



Electron micrograph drawings illustrating possible myonuclear derivation of myoblasts by dedifferentiation in the adult newt (Hay, 1979).

from the connective tissue, muscle and the skeleton at the distal portion of the stump. These blastema cells thereafter redifferentiated to form a new limb for the newt. The main point of this work was that during the formation of the blastema, the contribution made by muscle of mononucleated cells was by means of myonuclei dedifferentiating. This work strongly supported the theory that syncytial nuclei segregated cytoplasm of the myofibre and sequestered as mononucleated cells during newt regeneration as shown in the diagram above (Hay, 1979).

Furthermore it was reported that the adult newt had no preexisting satellite cells in the muscle. Hay (1979) concluded that muscle formation in the embryo by myoblast fusion was reversible in the adult newt when the need for myoblast proliferation was imposed on a muscle. Also, that there was enough reason to research for this myonuclear derivation of cells in avian and mammalian muscle. Hay (1979) felt that an important message from the newt ought not to be ignored by concluding that satellite cell was a reserve cell without which muscle regeneration was not possible.

2.4.1.2.2 Myonuclear derivation of myoblasts in injured muscle

The overall picture of muscle degeneration and regeneration was complex and this was attributed to the varying rates of morphological changes that occurred in the different parts of the injured muscle and to the different cells that prevailed in the area of the injury (Allbrook, 1962). Allbrook (1962) in his study reported bizarre multinuclei in myofibres of the injured muscle and this was attributed to infoldings of the nuclear membranes. Observation of 'sarcoplasmic phagocytosis' led to the strong presumptive evidence that the phagocytes were derived from muscle nuclei with a cytoplasmic envelope.

Allbrook, in the same study, also described mononucleated cells having varying degrees of basophilia and nuclear density. He went on to conclude that mammalian striated muscle fibres regenerated from pre-existing muscle nuclei which gained a cytoplasmic envelope and that these cells also engaged in phagocytosis.

Another study (Teravainen, 1970), using muscle after slight compression injury to avoid degeneration, further supported the myonuclear derivation of cells during regeneration. In this study, electron micrographs revealed areas of protoplasmic contact between satellite cells and the myofibre sarcoplasm. The conclusion in the above study was that satellite cells originated from myonuclei by pinching off from the sarcoplasm. According to Teravainen, these satellite cells had a pale nucleus with a prominent nucleolus and few organelles in the pale cytoplasm which also contained a number of pinocytic vesicles on the peripheral margins.

2.4.1.2.3 Myonuclear derivation of myoblasts in denervated muscle

Hess and Rosner (1970), working with denervated muscle of adult guinea pigs, were of the opinion that satellite cells increased in number by the dedifferentiation or budding mechanism described by Hay (1959/1979) for regeneration in the muscle of the newt. These workers described nuclei of myofibres being surrounded by a small amount of cytoplasm which separated from the experimentally denervated muscle fibre. They went on to state that the budding mechanism in the guinea pig muscle fibres seemed to appear without any specific distribution. Furthermore, they did not observe any mitotic division in the myofibres.

2.4.1.2.4 Myonuclear derivation of myoblasts in ischaemic muscle

Investigations on experimentally induced ischaemia in mice and rabbit muscles by ligatures, supported the concept that mononucleated cells that formed myoblasts during muscle regeneration are formed from pre-existing myonuclei (Reznik, 1969). Reznik (1969) also described myonuclei around which parent sarcoplasm was partitioned such that a new cell formed, being pinched off from the parent myofibre. According to Reznik, the surrounding cytoplasm of the myonuclei had different ultrastructural features from that of the undamaged region of the same muscle. He went on to suggest that the presence of satellite cells in adult myofibres represented transient morphological and functional stages during the development of new myogenic cells. Because many of the subsarcolemmal cells which were presumptive myoblasts were all called satellite cells led to a lot of confusion (Reznik, 1976). Reznik (1976), having compared research results in his review, was of the opinion that myoblasts could be derived from pre-existing satellite cells. But he found it difficult to accept that, ***in vivo***, so many myoblasts resulted from so few satellite cells in such a short time after local injury. Therefore, Reznik (1976) concluded that myoblasts were probably produced from other sources in the myofibre.

2.4.1.2.5 Myonuclear derivation of myoblasts in radioactive thymidine labelled muscle

Whilst other radioactive labelling studies promoted the hypothesis that satellite cells gave rise to new myoblasts during muscle regeneration, Walker (1972) using the same labelling techniques on rats was of the opinion that myotube nuclei were derived mainly from myonuclei. In the above study,

one group of rats was subjected to a single injury followed by radioactive thymidine injection. The second group was subjected to a double injury, after the initial injection of labels. The absence of labels in the myotube nuclei in the single injury group, together with the incidence of labelling of the myotube nuclei and centrally located nuclei of maturing fibres in the second group led to Walker's conclusion that myonuclei were the precursor cells.

2.4.1.2.6 Myonuclear derivation of myoblasts by "Potocytosis"

Lee (1965) investigated regeneration in denervated muscle where the sciatic nerve in the thigh of rabbits and rats were sectioned. Apart from observing the usual mononucleated cells reported by others in a sublaminar position, cells at various depths within the myofibres were observed in the gastrocnemius muscle, denervated for longer than three months. According to Lee, EM evidence suggested that the cells in the periphery were formed by invagination of the plasma membrane at the two poles of the myonucleus with the membranes from either side meeting and fusing, cordoning off the new cell thus formed. Lee went on to state that the cells at the deeper levels of the myofibre were supposedly formed by invaginations of the plasmalemma which encircled a portion of sarcoplasm and its organelles, with or without a nucleus leading to new cells. Lee went on to suggest that cells may be formed by compressed sarcoplasmic reticulum enclosing and ejecting some intracellular structures. This mechanism of cell formation was said to occur in other tissue cells, and in cells of other members of the animal kingdom (Lee, 1965). This mechanism was called potocytosis by Meltzer, 1904 (cited in Lee, 1965).

2.4.1.3 Presumptive myoblasts from connective tissue and phagocytosis during regeneration

2.4.1.3.1 Myoblasts from connective tissue elements

Sloper and Partridge (1980) expressed the possibility of connective tissue cells contributing precursor cells for muscle regeneration. On the other hand, earlier workers (Adams et al., 1962) believed that muscle restoration was always the result of proliferation of sarcolemmal nuclei and that connective tissue elements did not participate in the process of regeneration.

According to Witkowski (1977), identification between fibroblasts and myoblasts in culture was not possible. On other hand, it was said that fibroblasts could be differentiated from myoblasts by the presence of extensive elaboration of endoplasmic reticulum and multiple Golgi complexes (Lipton, 1977b). Furthermore, Lipton (1977b) also expressed the possibility, that in less than optimal environment, myoblast morphological differentiation may be modulated or reversibly altered. Ontell (1977) reported the presence of mast cells and satellite cells in the sublaminar area in neonatal rat muscle. The part played by these mast cells in muscle regeneration was not known.

2.4.1.3.2 Phagocytic myoblasts

There was other evidence that myoblasts may engage in phagocytic activity (Carlson, 1973). Mastaglia and Walton (1971) stated that the characteristics of some of the cells seen in polymyositis having cytoplasmic inclusions and autophagic vacuoles made differentiation between myoblasts and macrophages extremely difficult.

Gilbert and Hazard (1965), inspecting biopsies of muscular dystrophy patients, suggested that myoblasts assisted in the dissolution of necrotic material during muscle degeneration and regeneration.

2.4.1.3.3 Phagocytosis

According to Reznik (1969), the only mononucleated cells that contained myofibrils were clearly macrophages that had phagocytized necrotic muscle fibre fragments, but went on to state that some macrophagic cells were derived from necrotic myofibres. Active degeneration and phagocytosis of muscle fibres and regeneration activity were shown to be wide spread in the clinical stages of muscular dystrophy (Hudgson et al., 1967) and in all forms of dystrophies (Pearce and Walton, 1962).

Phagocytes were reported to infiltrate bupivacaine-treated rat muscle within 2 days of injection and satellite cells also became prominent at about the same time (Bradley, 1979). Bradley also showed, in the bupivacaine-treated rat muscle, that regeneration in myofibres occurred side by side with necrosis and phagocytosis after 4 days, followed by extensive muscle fibre regeneration by the 6th day.

The cytoplasm of the invasive cells with long finger-like projections were more abundant and less dense than that of satellites (Maruenda and Franzini-Armstrong, 1978). These invasive cells were thought to be monocytes which slowly differentiated to macrophages (Franzini-Armstrong, 1979).

Trupin et al.(1979) considered morphological criteria of shape and position of cells in regenerating muscle unreliable in diagnosing the status of

sublaminar cells, i.e., whether they were myoblasts or phagocytes. According to Trupin et al. (1979), many of the forms identified as satellite cells by other researchers were early invading macrophages which were yet to be active phagocytes. Many viable fusiform cells, apart from pyknotic nuclear fragments, were seen by them to lie adjacent to injured myofibres in minced muscle regenerates of frogs and young rats. Some of these cells appeared to lie within the fibres. Furthermore, they were not able to distinguish between the early satellite cells and the macrophages. In addition, the fusiform cells below the basal lamina often showed extensive phagocytic activity, with phagosomes, lysosome-like dense bodies and large masses of fibrillar material. Minced muscle explants were treated with colloidal carbon by Trupin et al. (1979) to identify macrophages which in the experiment were found both outside and inside the basal lamina. They concluded from this study that undifferentiated invading macrophages were able to mimic the appearance of the early endogenous myogenic cells.

2.4.2 Myonuclei during regeneration

The discussion on myonuclei will include all nuclei in a sublaminar position in the muscle fibre.

2.4.2.1 Myonuclei of myofibres

Myonuclei varied in its chromatin content, morphology, arrangement and location within myofibres.

2.4.2.1.1 Euchromatic and heterochromatic

Myonuclei of myofibres in normal muscle were described as euchromatic, but heterochromatic myonuclei were not uncommon and these, when observed, were regarded as nuclei of satellite cells that had recently fused with the myofibre (Moss and Leblond, 1971). In the newt limb muscle, euchromatic myonuclei became heterochromatic during the process of dedifferentiation (Hay, 1979). In dystrophic muscle (DMD and polymyositis) euchromatin content of myonuclei were increased (Ishimoto et al., 1983).

2.4.2.1.2 Myonuclei in rows

Lash et al. (1957) demonstrated myonuclei, away from the immediate site of injury after about 3 days, in adult mice undergoing marked swelling. Furthermore, they observed short rows of nuclei with large nucleoli appearing close to the injury. The perinuclear cytoplasm was also shown to become basophilic. In addition, they described nuclei having conspicuous infoldings which they thought were due to a condition of functional activity or the result of contraction and compression. Lash et al. (1957) also failed to see any mitotic figures within the sarcolemmal tube.

A plethora of plump nuclei sometimes occurring in rows, having one or more nucleoli in basophilic myofibres were observed in the preclinical stages of muscular dystrophy (Pearson, 1962).

2.4.2.1.3 Myonuclei of irregular shapes

Irregular shaped and tightly packed nuclei were observed together with the absence of mitotic figures in myofibres regenerating after injury, and evidence of how the nuclei originated were not found (Lash et al., 1957). Allbrook (1962) also reported seeing these irregular multinuclei and suggested that they were due to infoldings of the nuclear membranes. Similar bizarre forms of nuclei were reported for mitochondrial myopathy with clinical characteristics of progressive muscular dystrophy in a Japanese female infant (Nagaura et al., 1990).

2.4.2.1.4 Central myonuclei

Centrally placed nuclei typically observed in dystrophic muscle were suggestive of various stages of regeneration (Walker, 1962). Pyknotic central nuclei were observed in muscle in the preclinical stages of dystrophy (Hudgson et al., 1967). Central myonuclei, commonly found in DMD muscle, signalled the presence of young regenerated myofibres (Schmalbruch, 1979).

2.4.2.1.5 Pyknotic myonuclei

Myonuclei of injured muscle of rats and rabbits became pyknotic and disappeared from the degenerating tissue (Reznik, 1969). Mendell et al. (1972) reported shrunken and pyknotic nuclei within the first two days after incubation of human muscle explants in clot cultures. Appearance of pyknotic nuclei was the most striking and first change observed in explanted fibres (Witkowski, 1977).

2.4.2.1.6 Myonuclear number and location

Myonuclei were often observed in close proximity to satellite cells (Sandset and Korneliussen, 1978; Schmalbruch and Hellhammer, 1977). According to Fischmann (1979) the nuclear number in myofibres was generally, roughly proportional to the length and width of the fibre.

2.4.2.2 Nuclei of sublaminar mononucleated cells

Schmalbruch and Hellhammer (1977) showed that a relationship existed between satellite cell number versus myonuclei number for a given volume of muscle such that if myonuclear number increased, the satellite cells would show a proportional increase. Nuclei of satellite cells were 10-15μ long and 2-5μ wide (Campion, 1984). With few exceptions, nuclei of satellite cells characteristically exhibited dense peripheral heterochromatin (Dubowitz, 1985). Nuclear membrane indentations and infoldings were sometimes observed (Takahama, 1983) but the general shape of the satellite cell nucleus was regarded as being oval to elliptical with prominent nucleoli which are usually observed in active satellite cells (Campion, 1984).

Ontell (1977/1979) observed some satellite cells with less heterochromatic nuclei and with nucleoli in muscle clusters of 2 day old rats. Undifferentiated cells with heterochromatic nuclei were also observed deep within the myofibre in rat muscle showing compensatory hypertrophy (Schiaffino et al., 1979). Nuclear euchromatin content of satellite cells in regenerating muscle was found to be higher than normal controls, and this indicated activation of the nuclear DNA (Wakayama and Schotland, 1979). Smaller nuclei of satellite cells were attributed to the quiescent nature of resting satellite cells and that the larger nuclei of myopathic satellite cells

was due to cells becoming activated (Watkins and Cullen, 1988).

2.4.2.3 Nuclei of myotubes and young myofibres

Mitotic division of nuclei in myotubes or myoblasts were not observed either, ***in vivo*** or ***in vitro*** (Gilbert and Hazard, 1965; Mendell et al., 1972; Carlson, 1973; Reznik, 1976). The nuclei and nucleoli were reported to be larger in young regenerative myofibres compared with those in the mature form in human muscle (Engel, 1979). Rumyantsev (1979) showed labelled nuclei within differentiating myotubes by 72 hours after [^{3}H]thymidine injection and this suggested myoblast fusion. Nuclei of more differentiated myotubes in regenerating frog muscle displayed deep infoldings of the membranes and polymorphism (Allbrook, 1962; Rumyantsev, 1979).

2.4.2.4 Myonucleation

During embryogenesis, myonucleation or multinucleation of muscle fibres were reported to take place by myoblastic cell fusion (Speidel, 1938; Fischman, 1979). Myonucleation or the increase in number of nuclei in the myofibres was attributed to mitotic activity of satellite cells (McConnachie et al., 1964). Moss and Leblond (1970) suggested that cells proliferating mitotically, subsequently fused with the myofibres promoting myonucleation. This was interpreted from experiments using [^{3}H]thymidine labelling (Moss and Leblond, 1970; Allbrook et al., 1971; Snow, 1977/1979). Mitotic studies revealed that divisions, in this way, occurred in free undifferentiated myoblasts but not in formed myofibres (Gilbert and Hazard, 1965; Shafiq et al., 1968). Cells found fusing at the end regions of young myofibres of skeletal muscle in mice indicated a possible way in which nuclei were added to growing fibres (Williams and Goldspink, 1971).

Proliferation of cells sprouting from cultured myofibres (Bischoff, 1975), from explants (Jasmin et al., 1984a) and from dissociated mononucleated cells supplied ample proof that satellite cells underwent mitotic activity. On the other hand, Carlson (1973) and Reznik (1976) in their reviews, reported the absence of mitotic division in sarcoblasts or multinucleated myotubes.

Godman (1957) in his study on regeneration and differentiation of rabbit striated muscle, suggested that amitotic nuclear division was responsible for myonucleation. According to Cooper and Konigsberg (1961) the assumption, that nuclei with dumbbell shapes and those with clefts were signs of amitotic division, would be erroneous. Nuclei, in their view, assumed these shapes as they moved through, perhaps, restricted areas in the cytoplasm.

2.4.3 Myoblast fusion and myotubes

Early work suggested that the young myotubes and myofibres grew by terminal expansions (Clark and Wajda, 1947). It is now well documented that myotube growth takes place by myoblast fusion (Carlson, 1973). Closely applied junctions or areas of membrane association called attachment plaques, which were fusion points between muscle cells, were described in chicken embryos (Trelstad et al., 1967). Myoblasts adjacent to each other were said to develop 'outpouchings' which were signs of fusion (Mendell et al., 1972). Sometimes cytoplasmic projections were observed being engulfed one by the other (Shimada, 1971).

Apparent fusion between mononucleated cells and basophilic syncytial mass, containing many vesicular nuclei, with parts of muscle fibres in DMD was demonstrated with the light microscope (Mastaglia and Kakulas, 1969).

Even though myoblast fusion was cell specific, records of heterotypic fusion of myoblasts between different animals and different cells within the same animal existed (Wakelam, 1985). According to Wakelam, the molecular factors guiding alignment of cells was unknown and there was suggestion that fibronectin played a part in this. At a physical level, vesicles and tubules increase in number between opposed pre-fusion myoblast membranes which come close together (Wakelam, 1985). According to Schmalbruch (1979), strings of mitochondria in myofibres were thought to mark the line of fusion between myofibres or, alternatively, they marked the site of splitting of myofibres. Fusion between satellite cells and possible necrotic fibre was also suggested (Nichols and Shafiq, 1979; Snow, 1979).

Fusion between myoblasts in cultures were apparently controlled by the position of the cell in the division cycle (Bischoff and Holtzer, 1969). According to Konigsberg (1971) the number of cells in culture, and not merely the proximity of cells was necessary for fusion, and that this implied some diffusion mediated control of the fusion process.

Delaporte et al. (1984) demonstrated that the time of myoblast fusion was delayed, and that the myotubes were smaller in DMD cultures when compared to normals. Acetylcholinesterase was thought to play a role in the fusion of myotubes (Tennyson et al., 1973). Increase in new myotube number by myofibre branching and myofibre splitting was suggested for regenerating rat muscle, injured with hot Ringer's solution (Schmalbruch, 1976).

2.4.4 Cytoplasmic and nuclear inclusions

Cytoplasmic and nuclear inclusions of the myofibre and satellite cells had variable morphology and seemed to depend on the metabolic status of the fibre or sublaminar cells (Dubowitz, 1985).

2.4.4.1 Nuclear inclusions

Banker (1975) observed nuclear bodies that were oval and others that were rod-shaped bundles of closely packed parallel fibres varying in size in dermatomyositis of childhood. Deep invaginations of the nuclear membrane trapping cytoplasmic contents within the nucleus was also reported in polymyositis (Mastaglia and Walton, 1971). Intranuclear membranous structures were observed in perinecrotic myofibres in frog sartorius muscle (Rumyantsev, 1979). Tubular (Tome and Fardeau, 1980; Bouchard et al., 1989) and filamentous inclusions (Coquet et al., 1983; Coquet et al., 1990) were observed in occulopharyngeal muscle dystrophy. Cultured muscle from occulopharyngeal dystrophy patients also revealed nuclear inclusions (Tome et al., 1989). Complex patterns of invaginated nuclei containing many thin actin-like filaments, aligned in parallel groups together with smooth vesicles, were observed in young mdx mice (Anderson et al., 1987).

2.4.4.2 Cytoplasmic inclusions

An array of cytoplasmic inclusions were identified in most muscle disorders (Papadimitriou and Mastaglia, 1982), with lysosomes and lipofuscin bodies being the most common. Some cytoplasmic inclusion bodies were regarded as secondary lysosomes (Witkowski, 1986). Lipofuscin and lysosomes were

observed in the sublaminar cells in a variety of animal species: mice, (Shultz, 1976); frog, (Trupin, 1976) and hagfish, (Sandset and Korneliussen, 1978). The purpose of and the relationship between lipofuscin and lysomes were unknown, although an increase in the number of lysosomes in cells suggested that they were likely to be invasive cells (Campion, 1984).

Numerous large lysosome-like dense bodies were demonstrated in the preclinical stages of dystrophy (Hudgeson et al., 1967) and in regenerating myotubes in dystrophic foci (Lipton, 1979). Dense bodies in close proximity to the nucleus of young fibres were identified as lipofuscin bodies (Mastaglia and Walton, 1971). These dense bodies, also observed in mdx muscle, were referred to as residual bodies (Anderson et al., 1987).

Large autophagic rimmed vacuoles (Dubowitz, 1985) with dense concentric lamellar structures, vacuolar spaces and bodies of various shapes and sizes were observed in occulopharyngeal dystrophy (Bouchard et al., 1989). Rimmed vacuoles were thought to be lysosomes (Villanova et al., 1993).

Clusters of myelin figures and filamentous inclusions were reported in distal myopathy (Nonaka et al., 1981; Matsubara and Tanabe, 1982; Isaacs et al., 1988). An abundance of concentric laminated bodies observed in children with weakness and hypotonia were supposedly attributed to organized aggregation of excessive actin filaments produced during fibre hypertrophy (Payne and Curless, 1976).

In some rare cases of exertional myalgia, especially of the gastrocnemius muscle, internalised capillaries in myofibres were identified, perhaps due to ischaemic conditions of the muscle on exertion (Isaacs and Badenhorst,

1992). These internalised capillaries may also be construed as cytoplasmic inclusions.

2.4.5 Mitochondria

Redifferentiating cells in the blastema of newts had numerous mitochondria (Hay, 1959/79), but small and few mitochondria with decreased quantity of cristae development were observed in satellite cells (Campion, 1984).

Shear (1979), causing subtle injury to the extraoccular muscle of albino rats by exposure to incandescent and fluorescent light energy, showed increased number of mitochondria in degenerating myofibres with Z line disappearance. The mitochondria had dense osmiophillic bodies, and longitudinal paracrystalline inclusions in the intracrystal space as well as between the outer and inner membranes (Shear, 1979).

Pleomorphic forms of mitochondria with crystal vesiculation, myelin figure formation, dense osmiophillic bodies and paracrystalline substances in bar formation were seen in a variety of disorders (Price et al., 1964; Papadimitriou and Mastaglia, 1982; Isaacs and Badenhorst, 1992). The mitochondria were sometimes decreased in number (Mastaglia and Kakulas, 1969) and swollen and degenerate in DMD (Atkin et al., 1991). Mitochondrial elongation in distal myopathy (Isaacs et al., 1988) and accumulation in degenerating diaphragm muscle fibres (Kimura et al., 1990) were also reported. Numerous, large and bizarre mitochondria were seen in occulopharyngeal muscular dystrophy (Pauzner et al., 1991) and in mitochondrial myopathy with clinical characteristics of progressive muscular dystrophy (Nagura et al., 1990). Similar mitochondrial abnormalities

prevailed in cardiac muscle of DMD patients (Wakai et al., 1988).

Gregory et al. (1968) demonstrated fusion of mitochondria in the flight muscle of the blow fly. Flight muscle of insects were considered suitable for muscle development studies as the sequence of development and senescence was completed in a matter of days (Gregory et al., 1968).

Mendell et al. (1972) reported the presence of numerous degenerate-appearing mitochondria which seemed to fill the cytoplasm of explants in culture.

2.4.6 Z Line

Z line distortions with streaming of Z line material was pronounced in the diaphragm muscle of Fukuyama type congenital muscular dystrophy (Kimura et al., 1990). Z line streaming and disorganisation were also observed in the muscle of patients complaining of exertional myalgia (Isaacs and Badenhorst, 1992). In some cases of dystrophy, Z line twisting was observed (Lichtig et al., 1993).

2.4.7 Golgi complex

The regenerating myofibre did not often reveal obvious evidence of Golgi complex (Allbrook, 1962). Numerous Golgi complexes were observed in satellite cells supposedly originating from myonuclei (Teravainen, 1970). The cisternae and tubule system of the Golgi complex were not always well developed in satellite cells and the prominence of these organelles also depended on the state of activity of the cell (Campion, 1984). On the other

hand, Hay (1979) reported well developed Golgi elements in redifferentiating cells in blastema of newts. Large amounts of Golgi apparatus were reported in multinucleated myotubes in cultured human muscle (Mendell et al., 1972).

2.4.8 T-tubules, Endoplasmic and sarcoplasmic reticulum

A tubular membranous network of hexagonal pattern observed in chick embryo breast muscle were regarded as special morphological elaboration of the T-tubule system (Ishikawa, 1968). According to Ishikawa (1968), many of the 'inpocketings' of the sarcolemma commonly described as caveolae or pinocytic vesicles were assumed to give rise to these networks. Elaborate and swollen granular endoplasmic reticulum (GER) were common in the cytoplasm of satellite cells (Nichols and Shafiq, 1979; Campion, 1984).

Granular endoplasmic reticulum (GER) are not common in the sarcoplasm of the myofibre. Instead, variable numbers of sarcoplasmic reticulum and T-tubules were present in the intermyofibrillar and subsarcolemmal spaces (Dubowitz, 1985). Terminal cisternae and T-tubules appeared swollen and disorganized in extraoccular muscle of albino rats exposed to incandescent and fluorescent light (Shear, 1979). Satellite cells with less heterochromatic nuclei, found in muscle clusters of young rats, had well developed endoplasmic reticulum and vesicles (Ontell, 1979). Dilated profiles of sarcotubular system were observed in the muscle of young mdx mice (Anderson et al., 1987)

Regenerating human muscle fibres were shown to have more smooth endoplasmic reticulum and poorly developed T-tubule system than mature

fibres (Engel, 1979). Wakayama et al. (1979) observed lamellar bodies within the cisternae of sarcoplasmic reticulum and the nuclear envelope of satellite cells. When cited, they commonly appeared in the sub-satellite areas in the myofibre (Wakayama et al., 1979).

2.4.9 Ribosomes

According to Larson et al. (1969) there was a consistent and orderly relationship between polyribosomes and myosin development. An helical chain of ribosomal arrangement was reported in human myoblasts in culture (Mendell et al., 1972). The number of free ribosomes, found in the cytoplasm of satellite cells, varied and depended on the physiological state of the cell (Campion, 1984), and ribosomal clusters may become prominent during the activated state of the cells (Schultz, 1976; Nichols and Shafiq, 1979; Wakayama and Schotland, 1979; Campion et al., 1978). Engel (1979) reported greater number of free and membrane attached ribosomes in young myofibres than in mature myofibres of human muscle.

2.4.10 Developing myofibrillar elements

The presence of developing myofibrils in myoblasts and myotubes was well documented (Carlson, 1973; Mastaglia and Walton, 1971; Tautu and Jasmin, 1982; Jasmin et al., 1984a&b). Developing myofibrillar elements were not present in satellite cells (Campion, 1984). In 2 day old neonatal rats some myotubes did not have myofilaments, and this made the distinction between satellite cells and myotubes difficult (Ontell, 1977). Myofibrils in DMD patients were distinctly thinner compared to the normal, due to loss of peripheral filaments, and this was most noticeable in the Z line region (Hudgson et al., 1967).

2.4.11 Microfilaments and microtubules

Microfilaments and microtubules were not uncommon in mononucleated sublaminar cells (Nichols & Shafiq, 1979; Campion, 1984).

2.4.12 Pinocytic vesicles

Satellite cells were commonly observed to have few to many pinocytic vesicles along their plasmalemma facing the basement membrane and the plasmalemma of the myofibre (Teravainen, 1970; Campion, 1984). Pinocytic vesicles were also observed in active satellite cells of DMD patients (Wakayama and Schotland, 1979).

2.4.13 Sarcolemma

The sarcolemma is comprised of the basement membrane and the plasma membrane or plasmalemma of the myofibre (Dubowitz, 1985). The basement membrane or the basal lamina, during early development of myofibre, was thought to envelope free myoblasts associated with the myofibre and that in this way satellite cells obtained their sublaminar position (Kelly and Zacks, 1969; Church, 1969; Ontell, 1974). During the early degenerative reaction of the injured muscle, the basement membrane remained intact but the plasmalemma was often disrupted (Reznik, 1969; Carlson, 1973).

2.4.14 Miscellaneous

With the incorporation of fibroblast growth factor in growth media, Smith

and Schofield (1994) claimed to have successfully cultured dystrophic and normal myoblastic cells continuously for 18 months. According to Smith and Schofield (1994), there was minimal loss of phenotype in the cells cultured with fibroblast growth factor (FGF), and that the cells ability to fuse at high serum concentrations of FGF was minimal. The mdx satellite cells, which were found sensitive to fibroblast growth factor, may explain the successful regeneration of new fibres, ***in vivo***, in the mdx mice (DiMario and Strohman, 1988; DiMario et al.,1989).

Studies on muscle cells from foetuses and adult human muscle suggested a potential involvement of low-affinity nerve growth factor receptor in muscle development and regeneration (Baron et al., 1994). According to Oh and Markelonis (1979), neurotrophic effects of a protein fraction isolated from adult sciatic nerves were thought to promote further differentiation and maturation of aneural muscle cells. Non-innervated muscle fibres were maintained for a longer period with the incorporation of nerve growth factor in the culture medium (Oh and Markelonis, 1979). A platelet-derived growth factor was also suggested to play a potentially important role in regulating proliferation of myoblast and muscle differentiation (Tidball et al., 1992).

Ashby et al. (1993), in their study on mice with peroneal muscular dystrophy and muscular dysgenesis, concluded that development of secondary myotubes depended on neurally evoked electrical activity of primary myotube to promote formation of secondary myoblasts.

Nathanson et al. (1978) demonstrated skeletal muscle metaplasia where skeletal muscle consistently formed cartilage when explanted onto bone matrix.

2.4.15 Conclusion

Although the origin of muscle precursor cells have not been conclusively identified to date, the current consensus that satellite cells, embryonic remnants, were responsible for regeneration of adult muscle stemmed from the studies performed before the 1980's. Research work dealing with the identification of the precursor cells has since been neglected. Therefore, whether precursor cells of muscle regeneration are derived from resident sublaminar satellite cells or from the myonuclei remain an issue of conflict and need clarification.

CHAPTER 3

MATERIALS AND METHODS

3.0 Introduction

This chapter is divided into Part I - Materials and Part II - Methods. Part I outlines in detail all the necessary requirements for successful skeletal muscle culture and EM work. Part II deals with all the experimental procedures adopted in this study for the culturing of hamster and human muscle explants, and for the subsequent EM investigation.

Previous methods used in earlier muscle regeneration studies have been discussed under literature review in chapter 2.

Part I: MATERIALS

3.1 THE CULTURE LABORATORY

The culture laboratory was a separate room with entry from an adjacent room and not from the main passage so that entry was restricted only to authorised persons. The ceiling was fitted with two 1,2 metre UV lamps for sterilization of the room by radiation for two hours, the morning before culture work. A red hazard light on the outside of the door indicated whenever irradiation was in progress to protect one against the harmful UV rays inside the laboratory.

To chemically control microbial flora in the laboratory, the work benches were cleaned with 70% alcohol before being used. The floors were vacuumed and then mop-washed with a non-toxic detergent solution of calcium hypochlorite (swimming pool chlorine).

The windows in the laboratory were permanently closed to keep out dust. The airconditioner was adjusted to maintain a slight positive pressure in the room so that opening and closing of the door minimised the entry of unconditioned air into the room.

All the essential requirements for the tissue culture work were kept inside the laboratory to avoid frequent opening of the laboratory door while work was in progress. The equipment housed in the laboratory included the laminar flow cabinet, CO_2 incubator together with gas supply, centrifuge, weighing balance, inverted microscope, cultureware and the reagents.

Sterile rubber gloves, face mask and a clean laboratory coat were worn during all the experimental procedures. Smoking and the consumption of food was not permitted in the laboratory.

3.2 REQUIREMENTS FOR STERILIZATION PROCEDURES

Sterilization was conducted according to the under mentioned procedures to achieve, as used in a microbiological sense (Pelczar and Reid, 1965; Pelczar et al., 1993), an environment free of living microorganisms or a so-called sterile environment.

3.2.1 Chemical sterilization

Chemical antimicrobial agents, used in the culture laboratory, were alcohol and calcium hypochlorite.

3.2.1.1 70% alcohol (ethanol)

This was made up in distilled water (see reagent preparation) in the dilution commonly chosen as bactericidal. Sterilization of all exposed surfaces of the work benches in the laboratory, including the stainless steel work surface of the laminar flow cabinet was effected by swabbing the surfaces with alcohol. The 70% alcohol was also used for sterilizing the decapitated hamsters used in the experiment as this agent is nontoxic. Absolute or strong solutions of alcohol are not recommended as antimicrobial agents as these only have a bacteriostatic effect (Pelczar et al., 1993).

3.2.1.2 Calcium hypochlorite

This was made up as a solution in distilled water by dissolving 1 gram granules per 25 litre of water. This dilution gives twice the strength used in the shock treatment of swimming pools. It is antibacterial and antifungal, and at the same time it is convenient and safe to use. After washing and mopping the floor with this solution, any excess that was left on the floor was conveniently removed by UV exposure provided by UV lamps in the room.

3.2.2 Autoclaving

Sterilization of glassware, stainless steel dissecting equipment and some culture fluids that are thermostable, was effected by steam under pressure. This is used as a practical and dependable method of sterilization over the years in most laboratories to date. The autoclave was operated at 15 lb of pressure per square inch, and for 20 minutes. With this setting of the autoclave, sterilization is effected at 121 °C.

3.2.3 Dry heat

Hot air oven was used to sterilize cleanly washed glass pipettes enclosed in copper canisters. A temperature setting of 160 °C for 2 hours was used. Watch glasses and glass petri dishes, washed and wrapped in tin foil, were sterilized in this manner.

3.2.4 Filtration

3.2.4.1 Membrane filtration

The membrane filters (manufactured by Schleicher & Schull) supplied with holders were sterile, non-pyrogenic and disposable. For small volumes, membrane filters in holders were used with a syringe. This technique of pushing fluid under pressure through a membrane of pore size 0,2μm was used mainly to sterilize solutions that were thermolabile such as culture media.

3.2.4.2 Millipore filter unit

For large volumes of aqueous media sterilization, sterile, non-pyrogenic and disposable 0,22μm filter units (Sterivex-GS) were used in line with a peristaltic pump.

3.2.5 UV-Rays

Two UV lamps emitting wavelengths around 265 nm were installed in the laboratory ceiling to effect sterilization of the laboratory. This wavelength has the highest bactericidal effect. UV lights were switched on for 2 hours to effect sterilization.

UV radiation was also provided under the hood of the laminar flow cabinet to sterilize its walls and the work surface.

3.3 LABORATORY EQUIPMENT

All the larger equipment used in our laboratory for tissue culture work will be detailed in this section.

3.3.1 Laminar flow cabinet

Bino Instrumentation - Laminaire : Bio-Hazard 4BH manufactured in South Africa: This is a vertical flow safety cabinet fitted with two high efficiency particulate air (H.E.P.A.) filters, one to clean the air moving onto the work

surface and the other to clean the final air released to the atmosphere. A pre-filter of 5μm pore size came fitted, to protect the H.E.P.A. filters which restricted particle size above 0,3μm giving 99,997% efficiency. The velocity of air flow was adjusted to 0,5- 0,6 m/sec. The cabinet was provided with a fluorescent light, and a UV lamp for sterilization. Also fitted inside the cabinet was a gas supply tap with an automatic cut out switch in case of power failure.

The laminar flow cabinet used was also designed to give the operator protection against hazardous biological material that may be used within the cabinet. The efficiency of the cabinet was checked before use by exposing nutrient and blood agar plates to the internal atmosphere of the cabinet and subsequently incubating them at 37°C for 5 days to check for internal atmospheric microbial contamination. The cabinet occupied a place in the laboratory away from air currents. Maintenance checks were performed 3-monthly by a service technician.

3.3.2 CO_2 Incubator

Bino Instrumentation - Laminaire Model 160 L manufactured in South Africa: This incubator control ranges were as follows:

CO_2	0 - 20%
Temperature	Ambient - 60°C
Humidity	Relative - 95%

The temperature was set to 37°C and the CO_2 to 5%. To prevent desiccation of incubated cultures, humidity was adjusted to 80%. The

water bottle of the incubator was always kept full with distilled water. All adjustment procedures were performed according to manufactures specifications.

The inside of the incubator was regularly swabbed clean with alcohol when not in use. Once a month, the inside of the incubator was decontaminated by means of a built in facility as a further precaution against the accumulation of microbial flora. The procedure was detailed by the manufacturers. The temperature was maintained at 83°C for 1 hour during this decontamination procedure.

Once in 3 months, incubator efficiency tests were conducted by a qualified service technician.

3.3.3 CO_2 and low pressure valve

Industrial grade liquid CO_2 without residue was supplied in a large tubular industrial gas tank. The gas was conveyed via a rubber tubing. The rubber tubing was connected to the incubator valve inlet and the tank via a two stage regulator and flow meter. A CO_2 low pressure valve adjustable to 5 psi at 4 litres/min. was used to regulate the supply of gas. A second tank of gas was always kept in reserve.

3.3.4 Sterilization oven

A general stainless steel oven capable of 300°C was used for dry heat sterilization of glass pipettes, beakers, watch glasses, flasks and measuring cylinders.

3.3.5 Weighing balance

Mettler PE 360 : This sensitive balance was capable of weighing material of 360 grams and functioned up to 3 decimal places. It was used to weigh out the powders used in the different media preparation.

3.3.6 Inverted microscope

The microscope (ELIZA-Tokyo) had a quadruple nose piece with 4X, 10X, 20X and 40X objectives. Coarse and fine co-axial controls were present. The inverted microscope was essential to view the cultured material within the culture flasks, which otherwise is not possible with the standard microscope. A low-positioned camera port was provided for holding a 35mm camera for photography.

3.3.7 Dissecting microscope

This dissecting microscope (manufactured by Carl Zeiss) was used to view the explants to expedite either cross sectional or longitudinal cuts of muscle explants to the required size for EM processing.

3.3.8 Peristaltic pump assembly

The peristaltic pump (Millipore, Cat.No. XX8020230) was designed to provide a constant flow rate of 85 ml/min. It was used to filter-sterilize aqueous solutions, such as culture media and buffer solutions, under pressure.

3.3.9 Handigas tank

A 7 kg Cadac cylinder was used to supply handigas for the Bunsen burner housed inside the laminar flow cabinet via a gas tap with an automatic cut out.

3.3.10 Liquid nitrogen tank

A 35 litre liquid nitrogen dewar (manufactured by Taylor-Wharton) was used for storage of live cultured cells at -196 °C for future use. At this temperature, the cells may be preserved for indefinite periods. In the handling of liquid nitrogen, care was taken to protect against personal injury. The dewar was housed in a cold room to minimise loss of nitrogen by vaporization.

3.3.11 Centrifuge

Hettich-Universal 11 : This was a bench centrifuge capable of 10,000 rpm, and of holding 4 X 20 ml Sterilin centrifuge bottles. All bottles were balanced with equal weight on opposite sides before centrifugation to avoid vibration due to imbalance. The vibration may result in ineffectual sedimentation of the centrifuged material.

3.3.12 Filing cabinet

A filing cabinet was necessary for keeping proper records of EM negatives, prints, embedded specimens and EM grids.

3.3.13 Semi-Automatic pipette

TEC NO MARA - Pipetboy : This is an electrically controlled blow and suction apparatus used in conjunction with a pipette. The Pipetboy was used to suck and dispense all liquid media inside the laminar flow cabinet. The apparatus was designed to hold variable size pipettes. It made pipetting inside the laminar flow cabinet convenient, and it minimised chances of contamination.

3.3.14 Waste bottle.

A large waste bottle, with a rubber stopper having an inlet and outlet tube, was used to collect liquid waste during the culture procedure inside the cabinet. The inlet and outlet tubes of the rubber stopper were connected in line, with polythene tubing, to a pasteur pipette on one end and the laboratory suction tap at the other. In this way, the collection of waste and residue produced during the experimental procedure was facilitated, minimizing the frequent use of pipettes in and out of the culture flasks.

3.3.15 Refrigerator

A double door refrigerator with a separate freezer compartment (Leonard - Double Door) was used for the storage of culture media that required refrigeration at either 4 $^{\circ}$C or -20 $^{\circ}$C.

3.3.16 Guillotine

A stainless steel guillotine (Ealing-USA) designed for the decapitation of

small animals, like the hamster, was used to bring about rapid death of the hamsters. The guillotine caused minimal recognizable stress and pain to the animal. Furthermore, the guillotine, instead of anaesthesia, was used in order to avoid the influence of the anaesthetic that might interfere with the early regenerative processes in the muscle.

3.3.17 Waterbath

A stainless steel waterbath (Labotec with Thermomix 1441 Regulator) set to 37°C was required to warm the culture media before use, and also to thaw frozen culture reagents and frozen subcultures.

3.3.18 pH meter

The pH meter (Orion Research Digital pH meter 611) was used to adjust pH of the culture media and the buffers. The electrode was sterilized with alcohol and rinsed with sterile buffer before use.

3.4 CULTUREWARE

a. Sterile 25 cm^3 culture flasks (Sterilin Products).

b. Sterile disposable pipettes - 1, 2, 5 and 10 ml (Sterilin Products).

c. Sterile inert, 5 and 20 ml plastic bottles. The 20 ml bottles have a conical bottom so that they may be centrifuged (Sterilin Products).

d. Sterile 3 ml screw top vials or cryotubes (Nunc product) for storage of cells in liquid nitrogen.

e. Sterile soda glass storage bottles designed for culture work and to be able to withstand autoclaving.

f. General dissecting equipment - stainless steel scissors, forceps, scalpel blade holders, scalpel blades and dissecting needles.

g. General laboratory pyrex heat resistant glassware - petri dishes, conical flasks (smaller ones with screw caps), beakers, measuring cylinders, slides, coverslips, large watch glasses and large 25 and 50 ml volumetric pipettes.

h. Disposable sterile pyrogen-free membrane filters, 0,2 μm (Schleicher & Schull)

i. Small, plastic, square dishes with lids for storage of small bottles to avoid tipping over in the refrigerator.

3.5 HAMSTER MUSCLE SAMPLES

5 Normal Syrian hamsters of variable weight, age and one of them being a female, were used in this project (see Table 1). The animals were obtained from the Bio-Medical Resources Centre of the University of Durban-Westville.

Table 1. Hamster Data

Hamster No.	Weight / grams	Age /weeks	Sex	Colour
1	156	24	F	Brown
2	72	6	M	Brown
3	85	6	M	Brown
4	130	18	M	Brown
5	75	6	M	Brown

The hamsters were chosen for this research project because the two centres (Institute of Neurology and Neurochemistry, London and The Department of Pathology, University of Montreal) where the culture techniques were learnt,

made use of hamsters in their research. The reason was that hamster muscle apparently demonstrated similar growth characteristics to that of the human under culture conditions. The guidelines for animal care and experimentation, as laid down by the Medical Research Council of South Africa, were observed.

3.6 HUMAN MUSCLE SAMPLES

The ideal would have been to obtain muscle biopsies from normal subjects, but this was not possible for obvious ethical reasons. Therefore, debrided but clean muscle samples were obtained from 5 patients undergoing surgery for conditions other than muscle diseases. Gluteal, tibialis, flexor digitorum, sternohyoid and rectus muscle samples, from different subjects of both sexes ranging between the ages 23 and 65 years, were used in the study (see Table 2).

Table 2. Patient data

Patient No.	Age-years	Sex	Disease Condition	Muscle
1	23	F	Pilonidal sinus	Gluteal
2	65	M	Vascular disease	Tibialis anterior
3	42	F	Hyper-pararthyroidism	Sternohyoid
4	37	M	Abdominal stab wound	Rectus
5	26	F	Cut left wrist	Flexor digitorum

3.7 CULTURE REAGENTS

In this section the source of the reagents, the preparation and use of each of them in the experiment will be given. All bottles containing reagent and media were adequately labelled to avoid confusion and accidents.

3.7.1 Sterile deionised double-distilled water (DH_2O).

3.7.1.1 Source : H_2O

Ordinary piped water from the tap was used.

3.7.1.2 Preparation : DH_2O

Not all water is the same. Its composition with regards to dissolved solids and endotoxins changes from place to place and from time to time. Therefore to obtain pure water for culture purposes, tap water was double-distilled, deionized and millipore filtered. Water obtained in this way was sterilized in the autoclave in 1 litre quantities in glass storage bottles.

3.7.1.3 Purpose : DH_2O

Distilled water was used in the preparation of the culture media, gelatin solution, salt buffer solutions and in the preparation of 70% alcohol.

3.7.2 70% Alcohol (ethanol)

3.7.2.1 Source : Alcohol

The 70% alcohol was made in the laboratory from absolute ethyl alcohol purchased from BDH Chemicals.

3.7.2.2 Preparation : 70% Alcohol

700ml of absolute ethyl alcohol was diluted with 300 ml of sterile deionised double-distilled water in a sterile measuring cylinder and dispensed into sterile 500 ml storage bottles.

3.7.2.3 Purpose : Alcohol

70% alcohol was used as an antiseptic to clean the work surfaces of the

laminar flow cabinet and the glass worktops of the bench. The decapitated hamsters were decontaminated by being immersed in alcohol before dissection. All sterile dissecting equipment were placed in a beaker containing alcohol before use and in between interchange of equipment during dissection and explant preparation of the muscle. Eggs used in the preparation of chicken embryo extract were also washed with alcohol which were then flamed to effect sterilization. Hands and gloves were washed with alcohol before working under the hood. It is safe, non-poisonous and non-pyrogenic to use, having minimal effect on the explant cultures.

3.7.3 Hank's balanced salt solution (HBSS).

3.7.3.1 Source : HBSS

HBSS was prepared in the laboratory.

3.7.3.2 Preparation : HBSS

HBSS without calcium and magnesium was the general choice in most culture laboratories.

To 1 litre of sterile deionized distilled water in a sterile conical flask, the following chemical compounds were added:

KCl	400 mg
KH_2PO_4	60 mg
NaCl	8 g
$NaHCO_3$	350 mg
Na_2HPO_4	90 mg
Glucose	1 g
Phenol Red	20 mg

The chemical ingredients were dissolved with the assistance of a magnetic stirrer. Thereafter, the solution was sterilized by membrane filtration using an in line peristaltic pump, and dispensed into 250 ml sterile storage bottles.

3.7.3.3 Purpose : HBSS

HBSS was used as a transient nutrient medium to hold the muscle specimens immediately after their isolation from the animal. The buffer was also used to wash the explants free of debris which formed in the course of their preparation. Whenever the explants were transferred from one flask to a new one, to harvest the mononucleated cells grown in the original flask, buffer was required to wash the mononucleated cells before treatment with trypsin/versene solution. This was done to ensure that all serum and other protein residue of the previous nutrient medium was removed from the flask. In doing this, HBSS serves to irrigate the muscle tissue and the young cells supplying them with the basic inorganic ions, glucose and water to maintain their normal metabolism.

At the same time the physiological pH range of 7.2 - 7.4 is maintained. In order to prevent contraction of muscle cells, calcium and magnesium ions were not included in this buffer. Phenol red was added as an indicator. When the pH becomes alkaline, the solution turns pink-purple and orange-yellow when acidic.

3.7.4 Earle's balanced salt solution (EBSS)

3.7.4.1 Source : EBSS

EBSS was made up in the laboratory.

3.7.4.2 Preparation : EBSS

To 1 litre of sterile, deionized, distilled water held in a sterile conical flask the following chemical compounds were added:

KCl	400 mg
$CaCl_2.2H_2O$	265 mg
NaCL	680 mg
$NaHCO_3$	2,2 g
$MgSO_4.7H_2O$	200 mg
Glucose	1 g
$NaH_2PO_4.H_2O$	140 mg

After stirring to dissolve the powders, the solution was sterilized by membrane filtration using an in line peristaltic pump, and dispensed into 250 ml sterile storage bottles.

3.7.4.3 Purpose : EBSS

This was the buffer of choice used to make chicken embryo extract because of its high sodium bicarbonate concentration which compensates for the loss of CO_2 during the blending process of the chicken embryos. Thus pH changes were kept minimal.

3.7.5 Normal saline (0,85% NaCl)

3.7.5.1 Source : Saline

Saline was prepared in the laboratory.

3.7.5.2 Preparation : Saline

85 g of NaCl was dissolved in 1 litre of sterile, deionized, distilled water in an Erlenmyer flask. The saline was then sterilized by membrane filtration and dispensed into 250 ml storage bottles.

3.7.5.3 Purpose : Saline

Saline was used in the preparation of L-glutamine solution.

3.7.6 Dulbecco's modification of Eagle's medium (DMEM).

3.7.6.1 Source : DMEM

This DMEM with L-glutamine but without sodium bicarbonate was made by Flow Laboratories (Cat. No.1033120). The Powder medium was supplied in sachets to make up 1 litre of base medium.

3.7.6.2 Preparation : DMEM

Care was taken to make sure that the sachet with the powder was intact as powder media are hygroscopic, and on exposure to air may cake up resulting in loss of quality.

All the powder medium was added to 950 ml sterile, deionized, distilled water contained in a 1 litre sterile volumetric flask. This was performed at room temperature with gentle stirring. To this was added 3,7g of $NaHCO_3$ which is the most commonly used buffer in cell culture media because this is the main physiological buffer ***in vitro***. Water was again added to bring up to final volume followed by gentle stirring.

Its pH was then adjusted, using a pH meter, to 7,2 with either 1N NaOH or 1N HCl with gentle stirring.

The medium was then immediately sterilized by membrane filtration using a peristaltic pump under pressure to avoid the loss of CO_2. The medium was filtered into 500 ml storage bottles, adequately labelled and stored at 4 °C.

3.7.6.3 Purpose : DMEM

This is a chemically defined base medium which is supplemented with other additives to provide the optimal conditions for muscle cell cultures. The powdered media are formulated without $NaHCO_3$ to increase their stability and shelf life.

Phenol red in the medium provides an indicator to the pH status. If the pH becomes alkaline, the solution turns pink-purple. When acidic the colour will be orange-yellow.

3.7.7 Chick embryo extract (CEE)

3.7.7.1 Source : CEE

CEE - 50% in Earle's balanced salt solution without phenol red (Flow Laboratories -Cat. No. 2850146) was used in the preliminary culture work. Due to the high cost of the CEE, for subsequent experimental work, it was made in the laboratory according to the method provided by Prof. OA Hawtrey, former head of the Department of Biochemistry of the University of Durban-Westville.

3.7.7.2 Preparation :CEE

Bacterial and virus free Eggs of 9 days of incubation were obtained with the courtesy of Rainbow Chickens of SA.

The procedure for preparation of CEE was as follows:

a) The eggs were placed in egg holders with the big end up. Sterilization of the eggs was carried out by washing with alcohol and flaming.

b) The shell around the airsac was carefully removed with a pair of forceps and the membrane punctured, exposing the young embryo.

c) Sufficient embryos to weigh approximately 50 g were removed and placed in a sterile beaker (with predetermined weight),containing Earle's balanced salt solution without phenol red (EBSS).

d) After rinsing the embryos with gentle stirring, the salt solution was removed.

e) The embryos were then homogenised in a sterile blender for 30 seconds.

f) 1 ml of EBSS was then added for each gram of embryo and the mixture was homogenised again for another 30 seconds.

g) The mixture was then placed into Sterilin centrifuge bottles and incubated at 37°C for 30 minutes after which the bottles were centrifuged at 5000 rpm for 20 minutes.

h) The supernatant fluid was pipetted off and pooled in a sterile bottle to which 1 ml antibiotic/antimycotic mixture per 100 ml CEE was added.

i) Sterility tests were carried to check for contamination.

j) The CEE after labelling was stored at -20°C.

3.7.7.3 Purpose : CEE

CEE is added as an enrichment media to provide accessory growth factors or mitogens. Perhaps the CEE also provides a neural factor required to stimulate muscle cell proliferation, ***in vitro,*** in the absence of the nervous system.

3.7.8 Foetal bovine serum (FBS)

3.7.8.1 Source : FBS

Sterile FBS, virus and mycoplasma tested, was supplied by Whittaker M A Bioproducts (Cat. No. 14901B), and delivered frozen on ice in 500 ml bottle.

3.7.8.2 Preparation : FBS

FBS was placed immediately into the freezer at -20°C to avoid the serum thawing because frequent thawing renders sera less potent as a biological growth promoter.

When first required for use, the bottle containing the FBS was placed in a 37°C waterbath. After thawing, the serum was aseptically dispensed into 25 ml sterile bottles which were subsequently stored at -20°C, with the exception of 25 ml which was held behind for use. In this way, the FBS was not all thawed at each use but only a fresh 25 ml quantity thawed each time as required.

The thawed FBS sometimes necessitates centrifugation at 3000 rpm, before use, to remove the froth and precipitates that may develop with storage.

Before using the FBS for the experiment proper, its viability as a growth promoter in muscle cultures was tested on a preliminary hamster skeletal muscle culture. If proliferation of myoblast was not promoted by the FBS, the FBS would have to be discarded and a fresh lot ordered. The shelf life of most sera are known to be limited as advised by the suppliers.

3.7.8.3 Purpose : FBS

FBS is used in cultures as a biological growth promoter enhancing proliferation of cells, or myoblasts in this case. As such it must contain unknown accessory growth factors or mitogens. Therefore FBS, together with CEE, is used as a supplement to the base medium.

3.7.9 Horse serum (HS)

3.7.9.1 Source : HS

Sterile HS, mycoplasma tested, was supplied by Whittaker M A Bioproducts (Cat. No. 14403A), and delivered frozen on ice in a 100 ml bottle.

3.7.9.2 Preparation : HS

See preparation of FBS

3.7.9.3 Purpose : HS

In muscle cultures FBS is used for proliferation of cells while HS replaces it when prolonged maintenance of cultures is the requirement, as well as for the promotion of fusion of myoblasts to finally form multinucleate myofibres (Hauschka, 1974). It is thought that HS also promotes development of myofibrillar protein (personal communication - Dr. Tautu, University of Montreal).

3.7.10 Antibiotic-Antimycotic mixture (AA)

3.7.10.1 Source : AA

AA (100X concentration) was supplied by Gibco Laboratories (Cat. No. 0615245), in a lyophilized form.

3.7.10.2 Preparation : AA

The lyophilized AA (100X) mixture was reconstituted with 20 ml sterile distilled water to give final concentrations of 10,000 units Penicillin, 10,000 mcg Streptomycin and 25 μg Fungizone per ml. After gentle mixing to dissolve the powder, the AA mixture was dispensed in 2 ml quantities into 5 ml sterilin bottles and stored immediately at -20°C. This was done to avoid repeated thawing to prevent loss in activity of the AA mixture.

3.7.10.3 Purpose : AA

To control microbial activity in muscle cultures especially when cultures are maintained for protracted periods, sometimes for several weeks. The chances of contamination become increased because of the highly enriched nature of the final culture medium containing FBS and CEE.

The AA mixture was used in the proportion of 1 ml per 100 ml base medium. This gave concentrations in the final medium that provided broad spectrum activity, that is, a bacteriostatic and bactericidal effect against the growth of Gram negative or Gram positive bacteria and fungal elements. However, the concentrations are designed not to inhibit the growth of the cultured cells.

3.7.11 L-Glutamine (200 mmol)

3.7.11.1 Source : L-Glutamine

In the preliminary work, ready prepared L-glutamine (200 mmol) was supplied by Gibco Laboratories (Cat. No. 0435030) as 100X concentrate. For subsequent use 200 mmol L-glutamine was made up from stock glutamine, purchased from Gibco (Cat. No. 0661051H).

3.7.11.2 Preparation : L-Glutamine

2,923g L-glutamine was dissolved in 100 ml of normal saline to give a solution of 200 mmol L-glutamine (100X concentration).

This was sterilized by membrane filtration and stored in 10 ml quantities in sterilin bottles at -20°C. When required, 2 ml L-glutamine solution was added to 100 ml base medium (DMEM).

3.7.11.3 Purpose : L-Glutamine

L-glutamine, a requirement for growth of cells, is omitted from liquid media because of its instability in solution at temperatures above -10°C. Therefore it must be added to the culture media prior to use.

Even though powdered media had L-glutamine, base medium prepared from such powders necessitate the further addition of glutamine especially if the base medium was allowed to stand for some time.

3.7.12 Gelatin

3.7.12.1 Source : Gelatin

Gelatin in powder form was obtained from BDH Chemicals.

3.7.12.2 Preparation : 0,05% Gelatin

50 mg of gelatin powder was placed in a 500ml sterile conical flask containing 100 ml of sterile deionized distilled water.

The flask was closed with tin foil and autoclaved for 15 min. A large flask was used to avoid boiling over of the contents which froth on boiling.

The freshly prepared gelatin was cooled to approximately 50°C before use. The gelatin is used while warm or else it will gel when placed into the flasks, which are relatively cold, and therefore not spread evenly over the flask surface.

3.7.12.3 Purpose : Gelatin

The gelatin was used to gelatinise the culture flask. This gelatinisation promotes the adherence of young cells sprouting from the explant and it also promotes fusion of these cells to form myotubes. Otherwise many of the young cells will be found floating in the medium. Gelatin provides a form of matrix on which the cells grow.

3.7.13 Trypsin-Versene solution

3.7.13.1 Source : Trypsin-Versene

Trypsin-Versene was supplied by Whittaker M A Bioproducts (Cat. No. 17161), delivered frozen on ice in 100 ml bottle.

3.7.13.2 Preparation : Trypsin-Versene

The formulation of trypsin-Versene was as follows:

All items used are in mg per litre DH20

Dextrose	1000
KCl	400
NaCl	8000
$NaHCO_3$	580
Phenol Red	20
Trypsin	500
Versene(EDTA.2Na	200

3.7.13.3 Purpose : Trypsin-Versene

Versene, a chelating agent, was used to remove calcium and magnesium ions from the culture medium while trypsin, by its digestive properties, served to dissociate or free the cells adhered to the gelatinised flask.

3.7.14 Dimethyl sulphoxide (DMSO)

3.7.14.1 Source : DMSO

This was purchased from Unilab - SaARchem.

3.7.14.2 Preparation : DMSO

A 10% solution was made in growth medium.

3.7.14.3 Purpose : DMSO

DMSO was used as a cryoprotective agency. This minimized water retraction from cells during freezing to low temperatures and protected against cell fracture on reconstitution for subcultures.

3.7.15 Complete growth medium (GM)

3.7.15.1 Source : GM

The GM was made up fresh for each batch of cultures.

3.7.15.2 Preparation : GM

The formulation of the culture medium was that adopted by Jasmin et al. (1984), the only difference being that an increased quantity of FBS of 20 ml instead of 15 ml was used to boost growth and proliferation of young cells in the explants. FBS, CEE, AA and L-glutamine were all thawed in a 37 °C

waterbath before being added to the base medium at room temperature. The formulation of GM was as follows:

DMEM	100 ml
FBS	20 ml
CEE	5 ml
L-glutamine	1 ml
AA	1 ml

The medium was made up in special culture bottles designed for the purpose and stored at 4 °C.

The medium, before being used for the experiment, was tested for growth promoting quality by culturing myoblasts obtained from previous primary cultures that were stored in small vials in liquid nitrogen. GM was warmed to 37 °C in a waterbath before use.

3.7.15.3 Purpose : GM

GM was needed for initial hamster skeletal muscle cultures from which proliferating myoblasts were harvested and stored in liquid nitrogen for future use in testing new batches of growth media and viability of new batches of sera purchased.

The same GM, freshly prepared when required, was used to maintain the explant cultures in the experiment.

3.7.16 Maintenance medium (MM)

3.7.16.1 Source : MM

MM was made up fresh in the laboratory before use.

3.7.16.2 Preparation : MM

The formulation was the same as for GM except that it did not contain CEE, and FBS was replaced by 8 ml HS.

3.7.16.3 Purpose : MM

The medium was designed to slow down proliferation of cells and at the same time provide the necessary nutrients. The MM containing HS is used to promote fusion of myoblasts and increase myofibrillar development in myotubes.

3.7.17 Diff-Quick Stain Set

3.7.17.1 Source : Diff-Quick Stain

This was purchased ready prepared from American Scientific Products.

3.7.17.2 Preparation : Diff-Quick Stain

The stain set comprised the following:

Fixative - 0,18% triarylmethane dye in 100% methanol.

Solution I - 0,1% Xanthene dye in buffer and 0,01% azide

Solution II - 0,125% thiazine dye mixture, 0,062% azure A and 0,062% methylene blue in buffer.

3.7.17.3 Purpose : Diff-Quick Stain

This is a versatile rapid staining method which was used to stain the live cultures in the flask. This is a modification of the Wright method of staining. The 3 step staining method was completed in 15 seconds. The cells in the flasks or on slides were stained with each solution for 5 seconds only. It makes clear differentiation between nucleus, cytoplasm and granules.

3.8 EM REAGENTS FOR PREPARATION OF EXPLANTS

3.8.1 Source : EM reagents

The reagents for EM work were prepared by the EM Unit of the Faculty of Medicine, University of Natal.

3.8.2 Prepared EM reagents

Standard reagents used routinely in the EM Unit were:

a) 5% Karnovsky's fixative (glutaraldehyde/paraformaldehyde mixture), (Karnovsky, 1965);

b) Na-Cacodylate buffer (0,2M) with an osmolarity of 340-350 mOsm;

c) Osmium tetroxide 1% in cacodylate buffer;

d) Ethyl alcohol 70%, 90%, 100% (dilutions in DH2O);

e) Propylene oxide;

f) Propylene oxide and araldyte mixture, 1:1;

g) Araldyte (resin; hardener; plasticiser and accelerator);

h) Uranyl acetate in 50% alcohol;

i) Lead citrate, (Reynolds, 1963);

j) 1% toluidine blue.

3.8.3 Purpose : EM reagents

Karnovsky's fixative was chosen for the immersion fixation of tissue because it incorporates the rapidity of penetration of paraformaldehyde and the desired fixation properties of glutaraldehyde such that the structural integrity of the cells are maintained as near as normal as possible without distortion of cell size and destruction or leakage of components especially proteins out of the cells.

Na-cacodylate buffer was used to wash of fixative from the tissue, causing minimal shock to the cells. The buffer operates within the physiological pH range 7.2 - 7.4 at a calculated osmolarity of the buffer.

Glutaraldehyde as a fixative has the disadvantage in that it does not preserve lipids which are an important and integral part of membranes and cytoplasmic contents. To overcome this loss of lipids osmium tetroxide, which fixes lipids, was used as a secondary fixative to give the tissue extra stability and contrast.

Increasing concentrations of alcohol were used to wash and gently dehydrate the tissue for subsequent penetration by the embedding medium which is not miscible with water.

Propylene oxide, a highly volatile fluid with low viscosity, was used as a miscible intermediary between the alcohol and the embedding resin araldyte.

Araldyte as an embedding medium, was the choice at the EM Unit because it is not too brittle. It also gives good contrast between the tissue and the resin with subsequent staining. After clearing the tissue of alcohol with propylene oxide, pre-infiltration of the tissue with the viscous araldyte was achieved with a 1:1 mixture with the low viscosity propylene. This was followed by infiltration of the tissue with 100% araldyte.

Uranyl acetate and lead citrate are generally used as stains for the sections on grids. These heavy metal stains give good contrasts so that subsequent photography reveals clear morphological structures of the tissue under study.

Part II: METHODS

3.9 CULTURE PROCEDURE

Strict aseptic techniques were adhered to for all procedures involved in the culture work. Dissections and culture preparations were performed in the laminar flow cabinet which provided a sterile environment. All culture media and cultureware used were sterile. They were properly labelled to maintain precise records.

3.9.1 Gelatinisation of culture flasks

All culture flasks were gelatinised before use. 3 ml of 0,05% gelatin solution was pipetted into each flask and wetting of the total base area of the flask was ensured by gentle tilting, to and fro.

The flasks were then placed in the refrigerator at 4 °C for 2 hours, for gel to settle. Thereafter the excess gelatin solution was removed from the flask with a suction pipette to the waste bottle.
One flask was placed overnight in the incubator at 37 °C to check for sterility of the flasks. The other gelatinised flasks were placed in the fridge for use on the following day.

3.9.2 Testing of FBS

All sera were first tested for their viability to promote growth in cultures. If the serum used did not promote growth, as it happens with some batches that stay stored for long periods on the shelf of the supplier, new serum will need to be obtained.

Initially, a hamster had to be sacrificed and the test was carried out using its skeletal muscle. Adult hamster muscle was routinely used for these tests as it was found that muscle from this species responded, ***in vitro***, in a manner similar to that observed for human muscle (Rose Yasin, Institute of Neurology and Neurochemistry, University of London - Personal Communication). Subsequent testing of sera for viability as a growth promoter was conducted on mononucleated cells that were cryopreserved in liquid nitrogen (see **3.9.6** and **3.9.7**).

3.9.2.1 First primary hamster muscle explant culture

The above was performed as follows:

a) The hamster was rapidly decapitated with a guillotine, care being taken to cause as minimal stress as possible to the hamster before death.

b) After decapitation, the hamster was immersed in a beaker of 70% alcohol to ensure surface sterilization.

c) The excess alcohol was removed from the animal by swabbing with sterile paper towel.

d) The skin around the thigh was surgically removed and the thigh muscle was carefully dissected avoiding unnecessary adipose tissue.

e) Approximately 500 mg of thigh muscle removed, was placed in cold HBSS containing 2% AA in a sterile petri dish.

f) The muscle was sliced into smaller pieces with a sterile scalpel blade.

g) All visible fat and connective tissue were teased out with a pair of sterile needles.

h) These muscle pieces were then transferred to a sterile watch glass balanced on an open petri dish.

i) The muscle pieces were washed twice, each time with 5 ml HBSS + AA which was subsequently removed by suction.

j) After adding 3 ml GM, the muscle was then minced with a blade into approximately 1 mm^3 pieces.

k) More connective and adipose tissue that became visible after the mincing process were removed.

l) The GM was removed by suction with care being taken not to lose any of the minced muscle to the suction pipette.

m) The minced muscle was washed once again with GM to remove all remaining blood cells and debris.

n) After dispersion of the muscle mince in 3 ml GM, approximately 150-200 mg of muscle pieces or explants were then transferred with a large bore sterile pipette into each of two gelatinised culture flasks.

o) The explants were fed with 7 ml GM warmed to 37 °C. Care was taken to ensure that the flask was not over filled with medium to the level of the neck of the flask.

p) Gentle agitation of the flasks ensured even dispersion of the explants before they were incubated in air containing 5% CO_2 and 80% humidity at 37 °C.

q) Each culture flask was gently agitated once a day to prevent adhesion of the explants to the gelatinised surface of the flask.

r) Cultures were inspected every morning with the inverted microscope and observations were made for morphological changes, and to certify the absence of microbial contamination.

s) Old GM was replaced by new GM (warmed to 37 °C) on the 5th day of incubation.

t) After 7 days, when large colonies of young cells accumulated, adhered to the gelatinised surface of the flask, the explants were transferred together with the GM to 2 new flasks.

The young cells in the original flask were the first batch of primary cells obtained.

3.9.2.2 Fusion test to confirm presence of myoblasts

The above was performed as follows:

a) The original flasks with the first lot of primary young cells or presumptive myoblasts were fed with 7 ml MM after removing the all the old GM by suction.

b) These flasks were incubated for another 3 days by which time fusion of cells occurred, forming multinucleate myotubes.

This proved that a large number of the cells that sprouted from the first primary explant cultures were young muscle cells or myoblasts. This test confirmed that the FBS incorporated in the GM was viable for muscle cultures.

3.9.3 Second and third primary cultures

The above was preformed as follows:

a) The explants from the first primary (1°) culture that were transferred to the 2 new flasks were fed with fresh GM and incubated again, but this time for only 4 days.

Fewer days were now sufficient time for a confluent growth of young cells

as growth of cells was already generated during the first 7 days of incubation. These constituted a second lot of 1° cultures.

b) The explants from the second 1° cultures were transferred once more to 2 new flasks, fed again with fresh GM and incubated for another 4 days, these now being the third 1° cultures.

3.9.4 Harvesting of primary cell cultures

The above was performed as follows:

a) After removing the GM, the cells remaining in the second and third lot of flasks were washed with HBSS to remove protein from the environment.

b) Each flask was then treated with 3 ml trypsin-versene to dissociate the cells from the flask. This was controlled under the microscope, care being taken not to expose the cells for more than 10 minutes, because prolonged exposure to trypsin may cause damage to the cell membranes.

c) The trypsin-versene with the cells from each flask was then pipetted into 6 ml of MM contained in 20 ml Sterilin bottles to neutralise the trypsin.

d) These bottles with conical centres were centrifuged at 2000 rpm for 5 minutes.

e) The supernatant was removed and the pellets of cells were gently resuspended in 3 ml GM.

f) This suspension of cells was either immediately subcultured to produce secondary cell cultures or prepared for storage for future use.

3.9.5 Storage of primary cell cultures in liquid nitrogen

The above was performed as follows:

a) The primary cell suspension in GM was pipetted (0,5 ml) into each of 6 screw top cryotubes placed on ice.

b) To each vial, an equal volume of 10% DMSO was added as a preservative to prevent damage when frozen.

c) The vials were first placed in the freezer at -20°C for 30 minutes, to avoid temperature shock to the cells.

d) They were then suspended above the level of liquid nitrogen in the dewar for 1 hour to achieve a temperature of approximately -70°C.

e) After this, the vials were lowered into the liquid nitrogen to be stored indefinitely at -196°C until required. These cells in storage were used for subculturing and testing new batches of media.

3.9.6 Testing new batches of growth media (GM)

Muscle cultures were performed at different intervals as it was not possible to work with more than 2 hamsters a day. This was on account of the time taken to prepare for the culture procedure and due to other time constraints. In the case of human muscle cultures, they were done whenever the specimens were available. This resulted in the preparation of new batches of GM at prolonged intervals. Therefore, the GM had to be tested for viability by subculturing the cryopreserved cells before use in the experiment proper. This is an essential procedure or else the life of a hamster or the precious muscle sample from the patient, together with all the hard work involved in the culture procedure for the experiment proper, would have gone to waste if at the end it was discovered that the GM failed to support growth.

3.9.7 Subculture of cells stored in liquid nitrogen

The above was performed as follows:

a) Two cryovials with viable muscle cells, removed from liquid nitrogen, were thawed rapidly in a 37°C waterbath.

b) The contents of the vials were pipetted into 5 ml GM contained in 2 centrifuge bottles. The GM served to revitalize the cells and to dilute and remove effects of the DMSO.

c) The bottles were then centrifuged at 2000 rpm for 5 minutes.

d) After centrifugation, the supernatant was removed and the pellet was resuspended in 2 ml GM.

e) The resuspended cells in the 2 bottles were transferred to 2 flasks, fed with GM and incubated overnight.

f) The proliferation of these subcultured cells indicated the viability of the newly prepared GM.

3.9.8 Hamster muscle explant cultures for the regeneration study

The procedure for the above was as follows:

a) Skeletal muscle, from each of the 5 hamsters used for the regeneration study, was obtained and prepared for culture in the same way as that described for the first primary hamster muscle explant culture (see 3.9.2.1).

b) For the regeneration study, the 2 culture flasks of muscle explants from each hamster was incubated for a period of 10 days.

c) Four muscle explants with apparent intact structure were removed from each set of cultures, every day, from day one to day 10 after incubation. The chosen explants were placed into fixative for EM study.

d) Pre-incubated or uncultured muscle explants, placed into fixative for EM study, served as controls.

e) The progression of growth and morphological changes of the explants in culture was monitored daily with the inverted microscope.

f) Photomicrographs were obtained of all pertinent changes.

The explant method of culture was obtained personally from the University of Montreal, Canada, and was the same as that used by Tautu and Jasmin (1982) and Jasmin et al. (1984a). The only difference was that the GM had 20 ml FBS replacing the horse serum used at University of Montreal. Foetal bovine serum was chosen for this study on regeneration because of the advice of Dr. Yasin of the Institute of Neurology and Neurochemistry, University of London, that FBS was more potent as a promoter of proliferation of cells than HS.

3.9.9 Human muscle explant cultures for the regeneration study

Debrided, but clean skeletal muscle, obtained from 5 patients from the surgical theatre at King Edward the VIII Hospital, was transported in 20 ml Sterilin bottles containing cold 10 ml GM. The Sterilin bottles were immersed in crushed ice contained in a polystyrene bag.

A delay of 2 - 4 hours was incurred before the human muscles were subjected to the culture procedure.

Human muscle was subjected to the same techniques described previously for hamster muscle explant cultures (see **3.9.2.1** and **3.9.8**).

In the case of the human muscle, the control specimens were placed into fixative for EM study without delay at the theatre.

3.10 PHOTOMICROSCOPY AND ELECTRON MICROSCOPY

In this section the procedures used in the preparation of thick and thin specimens sections, for light microscopy and EM investigation respectively, will be discussed. Care was taken to ensure correct numbering of specimens, negatives and prints.

3.10.1 Preparation of muscle for EM

All control muscle and incubated muscle explants, from both hamster and human, were subjected to a similar procedure.

The control muscle pieces and the experimental muscle explants were fixed using Karnovsky's fixative (pH 7,4) for 1 -2 h. The fixative was supplied by the EM Unit of the Medical School, University of Natal. After fixation, the muscle specimens were orientated and cut to appropriate size under the dissecting microscope. The specimens were then conveyed to the EM Unit for the rest of the procedure shown in the schedule on the following page.

Four resin blocks were prepared from each hamster and human muscle explant specimens from controls and, for 10 consecutive days, from the incubated cultures.

Schedule of procedures, adopted for EM preparation of the fixed muscle explants, showing hydration, fixation, hydration, dehydration and embedding.

Step	Solution	Temperature °C	Time
1	Buffer rinse - 0.2M Na Cacodylate	24	10 min
2	Buffer rinse - 0.2M Na Cacodylate	24	10 min
3	Post fixation in 1% osmium tetroxide in 0.2M Na Cacodylate	4	1 h
4	Buffer rinse - 0.2M Na Cacodylate	24	10 min
5	Buffer rinse - 0.2M Na Cacodylate	24	10 min
6	Dehydration - 70% ethanol	24	30 min
7	Dehydration - 90% ethanol	24	30 min
8	Dehydration - absolute ethanol	24	30 min
9	Dehydration - absolute ethanol	24	30 min
10	Propylene oxide	24	30 min
11	Infiltration - propylene oxide:Araldite(1:1)	24	30 min
12	Araldite	60	1 h
13	Araldite	60	1 h
14	Araldite	60	1 h
15	Araldite in capsules	60	24-48 h

3.10.2 Ultramicrotomy

After polymerisation of the resin blocks, 1 micron semi-thin sections for photomicroscopy were cut from each block with a Nova Ultratome using glass knives. The sections from each block were removed to a drop of water on a glass slide. This was heat fixed and stained with 1% toluidine blue. General areas of interest of the sections were identified using a Nikon FX-3 photomicroscope and photographs were taken.

The field of interest being identified, the block was trimmed removing expendable areas. Ultrathin, approximately 70 nm sections, cut and dropped onto water moats, were picked up onto 200 mesh copper grids.

3.10.3 Heavy metal stains - Uranyl acetate and lead citrate

The copper grids with the sections were stained with heavy metal stains to increase electron contrast. This was achieved with uranyl acetate followed by lead citrate for 2 minutes in each solution.

3.10.4 Electron micrographs

A grid prepared from each block was studied using a Zeiss EM10B Electron Microscope at 60 kV. Morphological structures of interest were captured on Ilford fine grain plate film at required magnifications. The negatives were developed and prints were made as permanent records for future reference.

3.10.5 Conclusion

The progressive ultrastructural changes occurring in the regenerating muscle explants in culture, on a day to day basis, were thus investigated. The findings from this investigation and the interpretations thereon are presented in the following chapters.

CHAPTER 4

RESULTS

4.0 Introduction

During the preliminary experimental work in muscle culture, cells from primary cell cultures were formed into pellets which were resin embedded for electron microscopy. Cells grown in the flask were also resin embedded ***in situ***, for electron microscopy. Although this work does not constitute part of the project for the thesis, the results are presented out of interest. Some of the electron micrographs of this preliminary work are presented in Figs. **1*A*** to **1*C***. The electron micrographs of the cells which were embedded, ***in situ***, in culture flasks exhibited fusiform or spindle shaped morphology with long tails of cytoplasm. The cytoplasm contained well differentiated organelles such as endoplasmic reticulum, mitochondria, numerous electron dense lysosomal bodies and vacuoles. The nucleus of the fusiform cells were elongated with dense granular nucleoplasm (Fig. **1A**). High magnification micrographs (Fig. **1C**) revealed the presence of primordial myofibrils and chains of ribosomes which apparently marked the development of ribosomes. Fusion between a number of the fusiform cells was also observed. The presence of the developing myofibrils and fusion between the adjacent cells confirmed that these cells were myoblasts. The pellets prepared from dissociated mononucleated cells revealed cells that were morphologically round with fine cytoplasmic projections. The nuclei of most cells (Fig. **1B**) demonstrated one or two prominent nucleoli. The cytoplasm was similar in structure to the fusiform cells, but the electron dense lysosomal bodies, for some unknown reason, were not observed in many of these round cell forms.

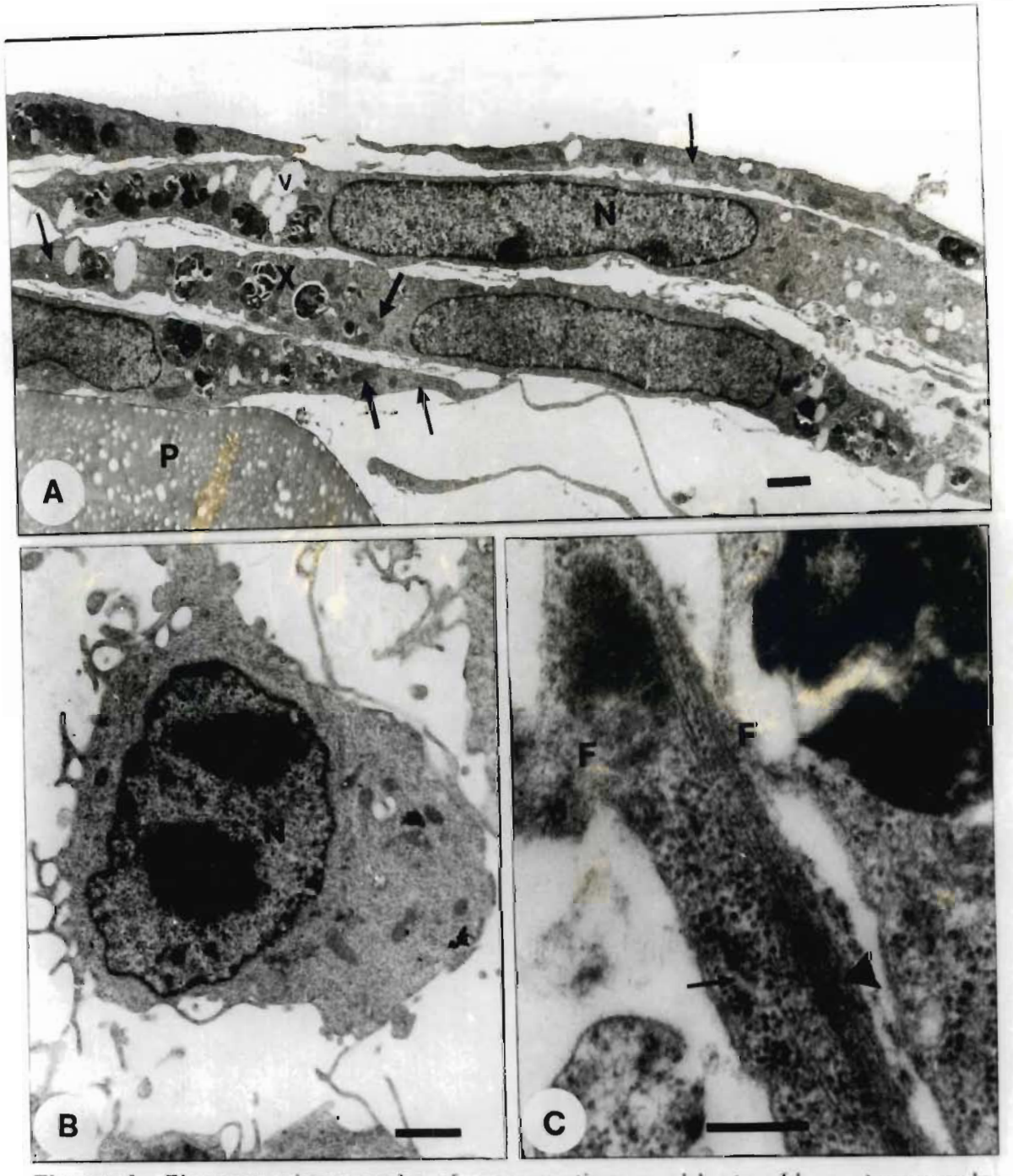


Figure 1. Electron micrographs of presumptive myoblasts of hamster muscle from mononucleated cell cultures. *A:* Fusiform, prefusion stage from dissociated mononucleated cells, embedded in, and sectioned direct from the culture flask. Cells contain vacuoles (v), cytoplasmic bodies (X), endoplasmic reticulum (thin arrows), mitochondria (thick arrows) and elongated nucleus (N). P, flask plastic. B: A typical presumptive myoblast from sections obtained from pellets of dissociated cells in culture. C: Part of a myoblast during the fusion stage from cultures as in *A*. Chains of ribosomes mark the development of endoplasmic reticulum (thin arrow). Developing myofibrils (arrow head) and fusion points (F) confirm the myoblastic status of these cells. Bar in *A* and *B* = 1 μm and 0,25 μm in *C*.

The morphological characteristics, observed in the pre-incubated control and the incubated explants in the experiment proper, are discussed under four major headings i.e. light microscopy of the hamster and human cultures with the inverted microscope, light microscopy of semi-thin sections of all resin embedded explants, electron microscopy of hamster explants and electron microscopy of human explants. The electron micrographs are presented in both chapters 4 and 5, in many instances to coincide with the discussion and to accomodate the thicker pages of the plates.

4.1 LIGHT MICROSCOPY OF CULTURED EXPLANTS UNDER THE INVERTED MICROSCOPE

4.1.1 Hamster explant cultures

Initially the explants were semi-transparent (Fig. 2*A*), floating in debris-free culture medium with the only contaminant being a few blood cells especially erythrocytes. The explants gradually became opaque from the 2nd day of incubation. The myofibres in some of the explants also appeared convoluted. The convolutions were due, perhaps, to the retraction of fibres which occur as a result of the mechanical manipulations during the processing of the explants. Myogenic cells or presumptive myoblasts (PM) migrating or sprouting from the muscle explants (Fig. 2*B*) were evident 3 to 4 days after incubation. Outlines of round forms appeared along the free margins of muscle fibres of most of the explants from the 4th day onwards, appearing as though cells were trying to push out from beneath the basement membranes of the myofibres. Some round cells were seen attached to the free margins of the explants. Many of these round cells

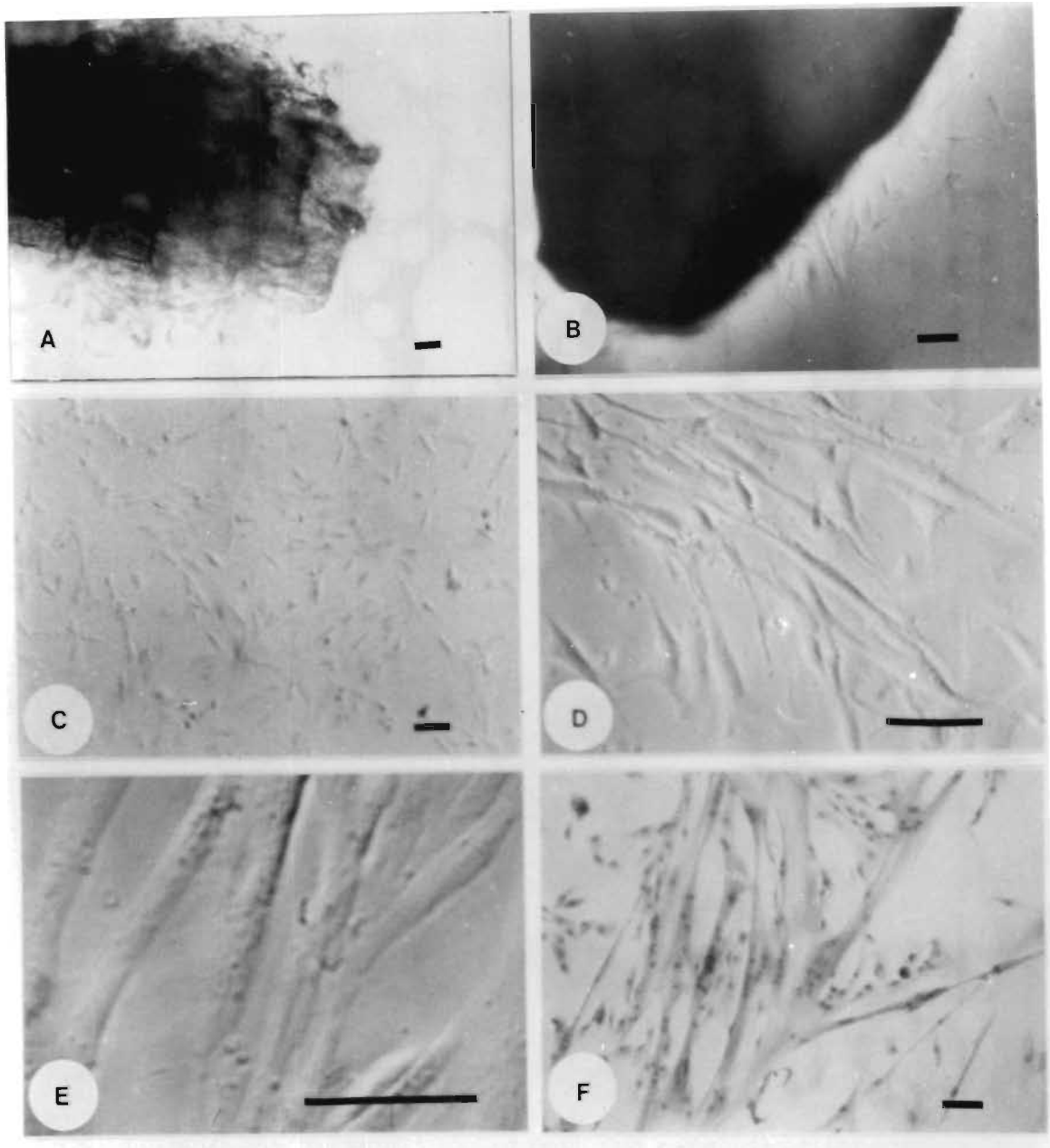


Figure 2. Photomicrographs of hamster muscle explant cultures. *A:* Part of single explant in culture flask, free of tissue debris and blood cells. *B:* Opaque explant, 5th day incubation, showing young mononucleated cells sprouting along the periphery. *C:* Prefusion, mononucleated, spindle shaped cells migrated from the explants onto the surface of the flask (6th day). *D:* Myoblasts fusing to form early myotubes which progressively become longer and multinucleated as in *E*. The nuclei are not visible as the above pictures were of unstained cultures. *F*: Multinucleate, branched myotubes and single presumptive myoblasts stained with Diff-Quick Stain Set. Cell fusion and multinucleation in myotubes are proof that many of the mononucleated cells or presumptive myoblasts were myogenic. Bar = 100 μm.

were also floating free in the medium. Cells that adhered to the gelatinised flask were spindle shaped and formed colonies (Fig. 2*C*). The spindle shaped cells in muscle cultures were possible myoblasts (Yasin et al., 1977). Some cells were pleomorphic with irregular cytoplasmic outlines. These, most probably, represented fibroblasts and perhaps also phagocytes freed from the blood capillaries. Even though the nutrient medium with FBS and CEE was chosen to promote proliferation, cell fusion (Fig. 2D) between cells aligned alongside each other due to the confluent growth, was observed. Fusion of the cells led to the formation of mutinucleate myotubes (Figs. 2*E*, 2F) from the 7th day of incubation. Nuclei were not clearly visible in unstained cultures but were identified within young cells only after staining (Fig. 2*F*). Fusion between the aligned cells and the formation of multinucleate myotubes confirmed the presence of myoblastic cells in the culture.

4.1.2 Human explant cultures

Light microscopy of cultures revealed that human explants behaved similar to hamster muscle, except that the human muscle fibres seemed more susceptible to retraction in the first few days of culture, and stabilised thereafter. Seeing that these specimens were obtained from patients prepared for surgery, it is possible that anaesthetics and other drug therapies, that the patients might have been subjected to, may have accounted, in part, for the fragile condition of the human muscle explants in culture. The migration of young cells from the explants were delayed when compared with hamster explants where the appearance of cells occurred as early as 3 to 4 days of incubation.

After 5 days of incubation only a few single cells were attached to the flask. Appreciable numbers of myogenic cells or presumptive myoblasts sprouting from the muscle explants, together with colony formation were evident between 6 and 7 days of incubation, indicating slower growth for human muscle in culture, as also reported by other workers (Jasmin et al., 1984). The fusion of cells to form myotubes was also delayed, being observed on the 10th day of incubation as compared to 7 days in the hamster. In addition, the growth of cells in the flasks in general was not as prolific as that found in the case of hamster muscle. Whether this was due to the effects of drugs and anaesthetics is not known. The myogenic potential of the cells was confirmed by the fusion of the cells to form myotubes. In general, the progression of growth observed in the cultures of human muscle explants was similar to that observed for hamster explants as shown in Figure 2.

4.2 LIGHT MICROSCOPY OF SEMI-THIN SECTIONS OF RESIN EMBEDDED MUSCLE EXPLANTS STAINED WITH TOLUIDINE BLUE

4.2.1 Semi-thin sections of hamster muscle

Semi-thin sections (Figs. 3A to 3*F*) revealed a steady increase in the number of nuclei with increase in incubation time. Uninucleate cell forms increased markedly after the 1st day of incubation. It was difficult to precisely distinguish between myonuclei and early uninucleate cells (Fig. 3*C*) at the light microscope level because the young uninucleate cells with scanty basophilic cytoplasm appeared morphologically similar to myonuclei.

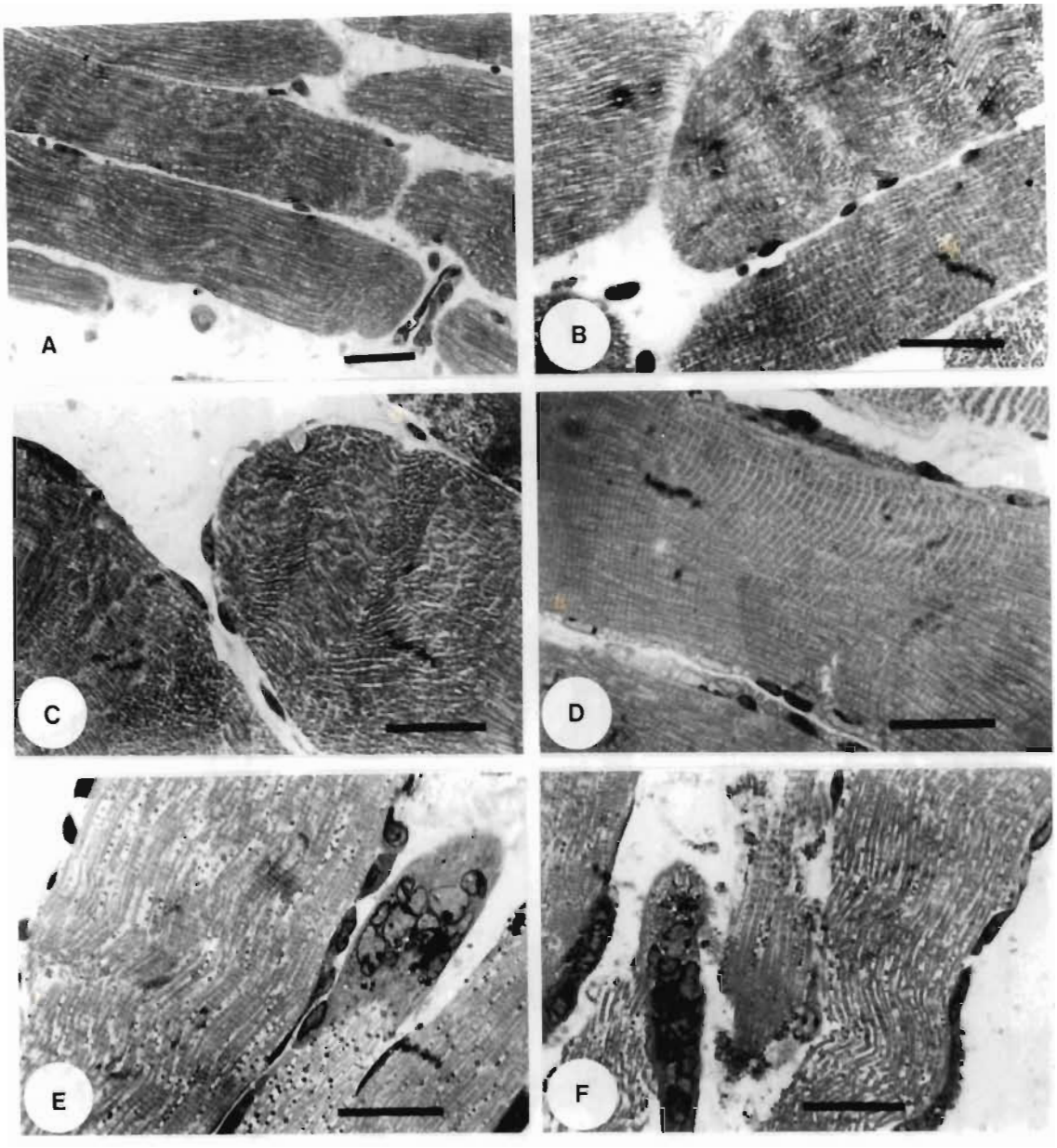


Figure 3. Photomicrographs of semi-thin sections of hamster explants, heat stained with toluidine blue. *A:* Pre-incubated control explant with few myofibre associated nuclei. *B:* 1st day incubation. Relative increase in pyknotic looking nuclei. *C:* 3rd day. Marked increase in myofibre associated nuclei some belonging to obvious cell forms. *D:* 5th day. Cell forms increased along the periphery of the myofibres. *E:* 7th day. More cell forms along the fibre and clusters of nuclei at the ends of some of the myofibres. *F:* 10th day. More clusters of nuclei and greater density of regenerative figures along the myofibres. At the light microscopic level it was not possible to precisely differentiate between myonuclei, mononucleated single cells or fused myotubes. Bar = 50 μm.

It was also difficult to deduce the characteristics of the intensely stained or seemingly pyknotic nuclei.

Multi-cells in chains or multinucleate cells developing along the myofibre (Fig. 3*C*) appeared from the 3rd day after incubation. Apparent cell forms together with clusters of nuclei, especially at the terminals of some myofibres, increased in number after 4 days of incubation (Figs. 3D to 3*F*). Again, it was not possible to differentiate whether each nucleus was present in independent cells or whether they belonged to the same syncytium.

4.2.2 Semi-thin sections of human muscle

Semi-thin sections (Fig. 4*A* to 4*F*) revealed a slow increase in the number of sublaminar nuclei with increase in incubation time. After the 1st day of incubation pyknotic looking nuclei showed an apparent increase in numbers (Fig. 4*B*). These probably were the heterochromatic myonuclei identified with the EM. An increase in the number of myofibre-associated nuclei were observed from the 3rd day of incubation. Some of the nuclei were centrally located and beaded in appearance (Fig. 4*C*).

Apparent sublaminar uninucleate cell forms increased steadily after the 4th day of incubation. Multi-cells in chains or multinucleate cells developing along the periphery of the myofibres appeared from the 5th day of incubation (Fig. 4D). More cell forms increased in number, with some cell forms showing large cytoplasmic content as incubation continued (Fig. 4*E*). Multinucleated cell forms and clusters of nuclei at terminals of some myofibres showed up much later in the human muscle cultures as compared with the hamster muscle (Fig. 4*F*).

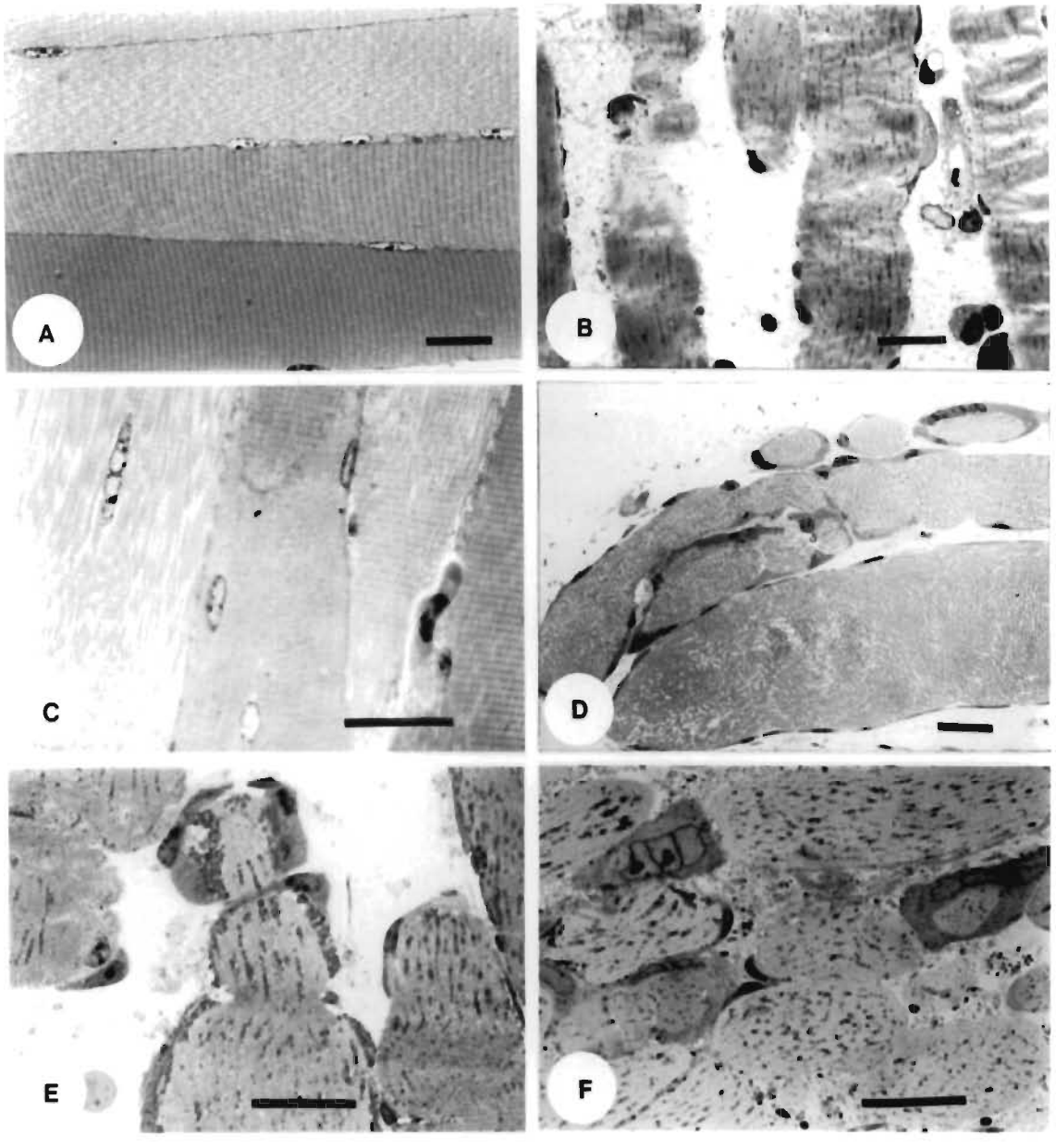


Figure 4. Photomicrographs of semi-thin sections of human explants, heat stained with toluidine blue. *A:* Pre-incubation control explant with myofibre associated nuclei. *B:* 1st day incubation. Relative increase in pyknotic looking nuclei. *C:* 3rd day. Marked increase in numbers of myofibre associated nuclei, some centrally located and appearing beaded. *D:* 5th day. Cell forms increased along the periphery of the myofibres. *E*: 7th day. More cell forms along the myofibre prevailed. *F*: 10th day. Similar to 7th day except that few clusters of nuclei appeared along the myofibres and at ends of some myofibres. It was not possible with the light microscopic to precisely differentiate between myonuclei, mononucleated single cells or fused myotubes. Bar = 50 μm.

Once again, it was not possible to differentiate with the light microscope, whether each nucleus was present in independent cells or whether they belonged to the same syncytium.

4.3 ELECTRON MICROSCOPY OF HAMSTER MUSCLE EXPLANTS

The electron micrographs recording the morphological changes occurring in the hamster muscle explants, during the experiment proper, are given below.

4.3.1 Controls: pre-incubation hamster explants

Ultrastructure of pre-incubation specimens which served as controls appeared normal with myofibre structural integrity remaining intact, keeping in mind the mechanical injury incurred during preparation of the explants. Myofibrillar disorganisation and the disruption of a few myonuclei were observed only in the ruptured fibres on the periphery of the explants which was obviously due to the mincing of the muscle.

Most of the myonuclei were typically euchromatic (Fig. 5*A*) as found in normal myofibres (Dubowitz, 1985). There were also myonuclei (Fig. 5*B*) with dense peripheral heterochromatin resembling the nucleus of satellite cells. The dense heterochromatic myonucleus shown in Figure 5B can quite easily be mistaken for a satellite cell. Perhaps the lapse of about 2 to 4 hours from the time the muscle was dissected from the animal to the time muscle explants were prepared was sufficient time to initiate changes in the myonuclei.

Typical satellite cells described by Mauro (1961) were observed in the control specimens (Fig. 9*A*). Some of the cells apparently appearing as satellite cells in the sublaminal region were not true satellite cells as they were attached at points to the parent sarcoplasm (Fig. 9*B*). Satellite cell counts, obtained by the number of cells below the basement membrane per 100 myofibre nuclei counted, ranged between 4% and 10% with the average being 7% (Table 3).

Table 3: Details of hamsters and the percentage of satellite cells in the control muscle.

Hamster Number	Age/Weeks	Sex	Satellite cells %
1	24	F	5
2	6	M	8
3	6	M	8
4	18	M	4
5	6	M	10

4.3.2 Day 1 after incubation - hamster explants

The structural integrity of the myofibres of the explants deteriorated with incubation time becoming oedematous with disorganised and degenerate myofibrillar elements. Much of the disorganisation of the myofibres could be attributed to the mechanical manipulation of the explant in the course of the experimental procedures. Nevertheless, myofibrillar and other cytoplasmic elements were always present in the myofibres for the full duration of the culture. Very few typical euchromatic myonuclei were observed. A consistent finding was that a number of myonuclei, after incubation, appeared more heterochromatic than euchromatic. Myonuclei

with a homogeneous distribution of dense granular nucleoplasm were also a common feature on day 1 of incubation (Fig. 5D). Often these electron dense nuclei were seen in the company of many mitochondria perhaps indicating their energy requirement (see appendix I and II). Approximately 3% of the myonuclei were obviously degenerate. While some of these myonuclei exhibited the disruption of the nuclear membranes with scattering of the nuclear contents to the outside of the myonucleus, others had shrunken nuclear envelopes with condensed chromatin which bore no semblance to either heterochromatin or euchromatin. This would represent the typical pyknotic nucleus described in some works (Snow, 1979).

Typical satellite cells were not observed in any of the incubated explants. The sublaminar mononucleated cells, observed in the explants after incubation, did not have the characteristic nucleus of satellite cells described by Mauro (1961). Many of the nuclei of these cells had diffuse, speckled and scattered chromatin (Fig. 9*C* and 9D). Some of the nuclei had nucleoplasm that was dense granular with a prominent nucleolus (Fig. 9*B*). After day one of incubation, all of these sublaminar cells had scanty cytoplasm. Some had cytoplasm at only one pole of the nucleus (Fig. 9D). There was no evidence of complete separation between the sarcoplasm and the cytoplasm of many of the young cells (Fig. 9*B* and 9D) (see Appendix I for more examples). The line up of vesicles often observed in the region between the young cells and the sarcoplasm, perhaps, served to form future membranes to separate the presumptive myoblast from the parent sarcoplasm as suggested by Hay (1959/1979). These sublaminar cells in their apparent formative stage with scanty cytoplasm associated with the nucleus reveal possible early stages of development of a presumptive myoblast from a myonucleus.

Even though the incubated myofibres appeared oedematous and showed degenerative changes similar to that described by other workers (Allbrook, 1962; Reznik, 1969; Baker and Poindextor, 1991), they still supported regeneration of presumptive myoblasts or young cells.

4.3.3 Day 2 after incubation - hamster explants

Typical euchromatic myonuclei were no longer observed. Myonuclei displaying invaginations of the nuclear membrane (Figs. 5*C* and 5D) were a common occurrence. Electron dense material was concentrated in the nucleoplasm proximal to the invaginations of these nuclei (Fig. 5*C*). Most of the other myonuclei were either heterochromatic or they contained electron dense granular nucleoplasm. The number of degenerate myonuclei did not show any apparent increase from that seen on day 1 of incubation. The only change was that the nuclear contents, within disrupted membranes of the myonuclei seemed to display disintegration and dissolution with ghosts of nucleoli visible in some of them.

There was a marked increase in the number of presumptive myoblasts within the myofibres. In some explants there were more myoblasts than myonuclei. From microscopical observations, it appeared as though there were greater number of myoblasts in the younger 6 week old than in the 18 to 24 week old hamsters. The presumptive myoblasts observed at this time fitted the morphological description of the so called activated satellite cells. They had greater amounts of cytoplasm than the characteristic satellite cells first described (Mauro, 1961). The cytoplasm showed greater differentiation with more organelle development. The presence of well developed endoplasmic reticulum, mitochondria, Golgi complex, microfilaments and

microtubules were clearly evident as differentiation progressed apparently with the decrease in the nucleus/cytoplasmic ratio of these presumptive myoblasts (Fig. 12*C and 13B*).

4.3.4 Day 3 after incubation - hamster explants

Myonuclei which were few in number were similar to those in Figs. 5B to 5D. The majority of sublaminal nuclei at this stage belonged to mononucleated cells or presumptive myoblasts.

Peripherally located uninucleate presumptive myoblasts with scanty cytoplasm, distinctly lying within the parent myofibre, were still present in the explants (Figs. 12*A* to 12*C*). Sarcoplasmic elements of the parent myofibre were clearly observed between the basement membrane and the myoblast cytoplasmic membrane (Figs. 12*A* and 12*B*), lending evidence that the myoblasts must be within the myofibre syncytium. The poor organelle development in these myoblasts with scanty cytoplasm reflects their immaturity.

The observation of remnants of sarcolemma in some of the parent myofibres, between the basement membrane and the myoblast cytoplasmic membrane (Figs. 12*B* and 12*C*), provides the evidence that the myoblast must have its origin from a myonucleus within the myofibre. Only parts of the sarcolemma of the parent myofibre were observed because of the obvious disruptive and degenerative changes the myofibre was subjected to during culture.

Myoblasts in chains and at different stages of development were present below the basement membrane along the length of many myofibres (Fig. 16*A*). Different stages of myoblast development were indicated by varying densities of cytoplasm and organelle development.

Cells in clusters were found at the distal ends of some fibres forming a cap (Fig. 16*B*). Binucleate and multinucleate myotubes also appeared in some explants. Mitochondrial aggregations (Fig. 16*B*) also made their appearance at this time, pointing to the energy requirement for the regenerative activity within the apparently degenerate myofibre.

Many of the cells, as they increased in number within the basal lamina, were aligned adjacent to each other promoting fusion of the cytoplasmic membranes between them to form mutinucleate myotubes (Fig. 16*C*).

The mitochondrial morphology of the parent myofibres showed changes even in some control specimens. There was evidence of some of the mitochondria undergoing obvious degeneration, showing crystal membrane damage and dissolution, leaving behind mitochondrial ghosts. In other instances, mitochondria revealed structural changes which were often reported for pathological tissue. Some of the mitochondria contained longitudinal bars of paracrystalline substance in the intercrystal space as well as between the outer and inner mitochondrial membranes (Fig. 14*A*). The presence of dense osmiophillic bodies in the mitochondria was a common occurrence. In other cases, mitochondria were large and swollen containing numerous vesicles inside them (Fig. 14*B*). Longitudinal and pleomorphic mitochondria were also observed in some explants.

Mitochondria in the intermyofibrillar space often appeared somewhat longitudinal, perhaps due to the compression on account of competition for space with the myofibrils.

Z line disorganisation and streaming were also observed in some intact myofibres (Fig. 14D). Many small smooth surfaced vesicles were often observed in the area of the Z line disorganisation.

4.3.5 Day 4 after incubation - hamster explants

Chains of myoblasts along the myofibres and clusters of cells at the distal ends still prevailed. Fewer single cells, isolated from other cells, were also present. Microfilaments, microtubules, endoplasmic reticulum, mitochondria and cytoplasmic ribosomes were now clearly evident in most of the mononucleated cells within the myofibre as well as in the myotubes.

An increased incident of fusion between mononucleate myoblasts occurred. Fusion between mononucleated myoblasts and myotubes with more than one nucleus was also observed. Multinucleate cells or myotubes were present in greater number along the myofibres (Fig. 18*B*). Early developing myofibrils were observed in the cytoplasm of these myotubes. Z lines were identified in some of the myofibrils which usually began to develop in the peripheral region of the myotubes.

Some of the cells were fusiform and others had cytoplasmic projections or pseudopodia resembling that of phagocytes (Fig. 18*B*). Some cells had phagosomal-like bodies with degenerate fibrils of the parent myofibre, and autophagic vacuoles in their cytoplasm (Fig. 18*A*), while others displayed

phagocytosis and or pinocytosis. Many of these cells (Figs. 18*A* and 18*B*) with phagosomes and those displaying phagocytosis had young, developing myofibrillar elements in their cytoplasm denoting their myogenic status. More aggregations of mitochondria were observed at the distal ends, as shown for the human explant (Fig. 15B), of the myofibre and in the subsarcolemmal regions (Fig. 23B). Intermyofibrillar mitochondria also increased in numbers, but not to the same extent as in the other areas mentioned.

4.3.6 Day 5 after incubation - hamster explants

Single cells of different morphological stages of development still prevailed in chains, in a sublaminar position within the parent myofibres (Fig. 16A). Proliferation of mitochondria was clearly apparent in most of the myofibres of the explants. Aggregations or clusters of mitochondria along the periphery and at the distal ends of some of the myofibres were evident (Fig. 16B).

In a number of cases where possible amitotic nuclear division was suggested, the cells involved were clearly identified as myoblasts or early myotubes because developing myofibrils were present in their cytoplasm.

Multinucleate myotubes with myofibrillar development were more apparent (Fig. 18B). Many of the multinucleate myotubes had well developed myofibrils. However, these young myofibrils did not fill up the cytoplasmic area of myotubes. Fully fledged young myofibres were not observed in any of the cultured explants. After multinucleation of the myotubes and the development of myofibrils, the myotubes at this stage failed to proceed to full maturity. The cytoplasmic organelles of these semi-developed young

fibres showed signs of degeneration. The presence of vacuolated ghosts of mitochondria and autophagic vacuoles increased in number in the cytoplasm of these partially formed mutinucleated myofibres.

4.3.7 Days 6 to 10 after incubation - hamster explants

The EM observations of morphological changes in the explants between 6 and 10 days incubation are presented together because the regenerative features were asynchronous. Leading on from the 5th day of incubation, similar changes were observed in the explants on different days of incubation. There was no precise cut off point in the various phases of development to state categorically that any one change occurred only on a particular day. This was probably due to different rates of developmental growth in different explants, attributable to age of the animals and perhaps the size of the cultured explants.

Many myoblasts which lay below or above myotubes (Fig. 16C), in some cases seen fusing with myotubes were still present within some parent myofibres. A large number of these myotubes showed degenerative features with degenerate mitochondria and autophagic vacuoles.

Dense mitochondrial clusters (Fig. 23*B*) appeared in a peripheral position along the subsarcolemmal regions of many of the parent myofibres. The basement membrane was convoluted with clusters of mitochondria packed below them, such that waves of mitochondrial clusters (Fig. 23*A*) lay along many of the parent myofibres.

Giant mitochondria were observed in some of the presumptive myoblasts that prevailed during this time of incubation (Figs. 33*A* and 33*B*). These giant mitochondria appeared to be a fused product of several mitochondria. Apparent fusion of a smaller mitochondrion with the giant mitochondrion is shown in Fig. 33*A*. The crystal membrane pattern was disorganised and the giant mitochondria appeared degenerate. Even if they were degenerate, a question that needs to be answered is the reason for the mitochondria undergoing fusion.

4.3.7.1 "New generation cytoplasm, nuclei and cells"

Observations from the incubated explants, from any one animal series, indicated that waves of mitochondria along the fibres seemed somehow to be replaced by cells with incubation time. Instead of seeing waves of mitochondria along the fibres, young cells were seen instead. These cells invariably had dense granular cytoplasm without much organelle development. Their nuclei varied in structure, appearing faintly granular to having clumps of electron dense material.

Electron dense mitochondria, with electron dense osmiophillic bodies, lying in the central regions of the myofibres were associated with undifferentiated plasmic forms (Fig. 24*A*). It appeared as though the plasmic material was leaking out of the mitochondria.

Also found in the central regions of some myofibres were strands of differentiated cytoplasmic forms (Fig. 24*B*). In the region where all these cytoplasmic strands were observed, there were no associated nuclei to suggest that these strands were pieces of cytoplasm belonging to a cell

below or above the plane of sectioning. Although, serial sections were not performed, one would have expected to see just a part of an established nucleus associated in at least one of the large number of cytoplasmic forms encountered in the study. Clusters of mitochondria together with some vesicular structures, partially bordered by cytoplasmic strands, were also observed (Fig. 25*A*). The vesicular structures were probably sarcoplasmic reticulum.

Differentiated cytoplasmic formations without nuclei were identified in the subsarcolemmal and central regions of the myofibres (Fig. 25*B*). These cytoplasmic formations had autophagic vacuoles, electron dense bodies similar to the electron dense mitochondria found outside them, and dilated endoplasmic reticulum. Nuclei were not seen associated with all these cytoplasmic masses identified. These cytoplasmic formations found associated with sarcoplasmic organelles mainly the mitochondria probably represent "new generation cytoplasm".

Some of the differentiated cytoplasmic formations contained fragments of membrane structures (Fig. 26*A*). These cytoplasmic formations were still attached to or intimately associated with mitochondrial and other sarcoplasmic organelles. Higher magnification of these membrane fragments revealed that these might be parts of a doubled nuclear membrane (Fig. 26*B*). These membranes were randomly distributed in the cytoplasmic masses and, therefore, it was unlikely that they belonged to degenerate nuclei. In extensions of some of the cytoplasmic masses (Fig. 26*C*) membranes were observed to partially cordon off material similar to granular nucleoplasm with many inclusions.

At the terminal parts of many myofibres, mitochondrial aggregations seemed to have been replaced by clusters of cells. At some terminals, large vacuoles were formed by cells lying above and below them (Fig. 27*A*). These vacuolated areas in between the cells contained sarcoplasmic organelles, mainly mitochondria. Amidst this mass of sarcoplasmic organelles, membranous forms drew incomplete patterns of irregular nuclei. Higher magnification of similar membranes in another explant from a different animal (Fig. 27*B*) showed the membrane structures to be similar to that of nuclear membranes. The appearance of chromatin-like patches within the partially enclosed membranes is suggestive of the formation of "new generation nuclei".

The "new generation cytoplasm" with the "new generation nuclei" probably gave rise to the many cells observed in the later incubation period. The areas previously occupied by mitochondrial aggregations and clusters seemed later to be replaced by cells which may be "new generation cells". Some of these cells were found lying above remains of the mitochondrial clusters (Fig. 28*A*). The terminal parts of fibres which had greater aggregations of mitochondria and other organelles would appear to give rise to numerous cells (Fig. 28*B*). The larger aggregations would appear to give rise to clusters of cells. These new generation cells had some morphological features in common, and this perhaps depended on their state of physiological maturity. The nucleoplasm was diffuse and granular with nuclear inclusions. The cytoplasm of these cells was dense granular with very little differentiation. Cytoplasmic inclusions were not uncommon.

Clusters of irregular nuclei were observed within a myotube at the terminal parts of some fibres (Fig. 29). The cytoplasm of these myotubes had

autophagic vacuoles and degenerate mitochondria. Large vacuolated areas adjacent to some of the nuclei appeared to be extended outer nuclear membranes or perhaps empty spaces left by nuclei that may have been lost during the EM processing. Multinucleated myotubes showing similar irregular nuclei were a common feature. Some of these myotubes with the irregular nuclei formed a cap over the terminal part of some myofibres (Fig. 30), and often extended down the length of the myofibres (Fig. 31). The nuclear morphology of many of these irregular nuclei gave the impression that they were either segmenting or budding off more nuclei. These myotubes with the irregular nuclei were below the basement membrane of the parent myofibre. Although the myotubes had many nuclei, the myofibrillar content of these myotubes were scanty.

4.3.7.2 "Generation workshops"

Some of the myotubes growing in a cone formation at the distal ends of myofibres (Fig. 34*A*) exhibited what appeared to be "generation workshops". A number of areas showing the possible derivation of structural elements identified in pathological muscle is marked as areas 1 to 4 in Fig. 34*B*. These areas are shown magnified in Fig. 35.

In Area 1, an electron dense cytoplasmic or lysosomal-like body is associated with a structure containing fine fibrous elements. Another structure adjacent to this contains a dense mitochondrion and a lysosomal body looking much like a degenerate mitochondrion at the opposite end of the structure. In between are found vacuoles with glycogen. The structure, wrapped around by what appears to be formative membranes, perhaps signals the beginnings of a new nucleus (Fig. 35*A*).

In Area 2, structures resembling partial formations of nuclei are shown. Incomplete membranes seem to border electron dense chromatin-like material (Fig. 35*B*). In Area 3, fibrous myelin-like bodies are associated with mitochondrial remnants in which the crystal formation is apparent. This appears similar to myelin or fibrous structures observed in pathological muscle (Fig. 35*C*).

In Area 4, again a degenerate looking mitochondrion seems to be involved with fibrous structures. Elongated electron dense structures showing some semblance to mitochondria are also present in this area (Fig. 35D).

4.3.8 Myonucleation - possible amitotic division

Nuclear morphology showing signs of amitotic division were observed 3 and 8 days of incubation. It is on this account that it is being presented separately.

Some of the nuclei, observed within the sublaminar presumptive myoblasts with well developed cytoplasmic organelles, showed signs of budding, splitting or segmentation (Fig. 20). Chromatin accumulation at the possible future segmentation zones seemed to herald the nuclear division (Figs. 20*A* and 20*B*). Bilobed nuclei were observed in some cells (Fig. 20*C*). The nuclear lobes were linked by an apparent thin strand of nuclear material, suggesting a possible future break between the lobes. Outer membranes of nuclear compartments of some myotubes appeared extended with multinuclei within. Many of these nuclei appeared as if they were budding. In some cells, the nucleus exhibited multilobes (Fig. 20D). The multilobulation seemed to occur within the expanded or vacuolated outer

nuclear membrane. The lobes of this nucleus (Fig. 20D) are linked by thin strands of nuclear material. Furthermore, it appears as if the smaller lobes at either poles of the nucleus were 'nuclear buds'.

4.4 ELECTRON MICROSCOPY OF HUMAN MUSCLE EXPLANTS

4.4.1 Controls: pre-incubated human explants

The control specimens or uncultured explants, showed some signs of early regeneration. This was probably due to the fact that these muscle samples came from sick patients and from sites of injury. The possible influence of anaesthetics and drugs on initiating some regeneration features in the muscle of these patients must be born in mind. The muscle specimens, taken direct into fixative for EM at the hospital theatre, were essentially intact with hardly any damage to the components of the myofibres. But the myofibres on the outer border of the muscle showed some retraction.

Some of the myonuclear morphology observed before the specimens underwent incubation are presented in Figs. 6 and 7. Apart from normal euchromatic myonuclei (Fig. 6*A*), many myonuclei (Fig. 6*B*) with dense peripheral heterochromatin, similar to those found in satellite cells, were observed. The electron dense heterochromatin indicated activation of the myonuclei. Some of the electron dense myonuclei were associated with large amounts of glycogen (Fig. 6*B*). Many of the myonuclei which showed signs of activation seemed to often have electron dense bodies in the near vicinity (Figs. 6*B* and 6D). In Fig. 6*B*, the electron dense body is closely applied to the nucleus so that it gave the appearance that the nucleus was

giving off a nuclear bud. These electron dense bodies often appeared vacuolated. Some activated myonuclei with diffuse granular nucleoplasm contained prominent nucleoli (Fig. 6D).

Indented and beaded forms of myonuclei in the explants of some of the patients were common (Fig. 6*C*). Centronucleation of some of the myonuclei indicates that they were probably young fibres going to maturity, indicating again that regenerative changes were already present in these so called 'normal' muscle. Many of the beaded appearing myonuclei (Fig. 7*A*) were apparently independent nuclei with a prominent nucleolus in each segment. Some myonuclei with deep indentations, on magnification, revealed lines of possible segmentation (Fig. 7*B*) suggesting the derivation of beaded myonuclei.

Myonuclei in control specimens were also observed imbibing myofibrillar elements (Fig. 7*C*). Typical double nuclear membrane, adjacent to the area of imbibition, were not present. These areas of the myonuclei were commonly electron dense. In areas of activity the Z lines disappear or become disorganized with obvious disruption of the myofibrillar arrangement. Many small vesicles were often present in the areas of imbibition. Electron dense bodies (Fig. 8D), referred to as residual bodies, associated with numerous small vesicles were seen in control and incubated specimens. They were also identified in hamster muscle explants. These electron dense bodies had no visible limiting membranes. They were usually found opposed to and involving the Z line material.

The satellite cell counts ranged between 0 and 7% with the average being 2.6% (Table 4). Many of the cells counted, strictly speaking, did not

conform to the characteristic requirements for classification as satellite cells. Typical satellite cells (Fig. 10*A*) were rare. Many of these cells were presumptive myoblasts in a subsarcolemmal position within the parent myofibre.

Table 4: Details of patients and the percentage of satellite cell counts in control muscle.

Patient No.	Age (yr)	Sex	Muscle	Satellite cell %
1	23	F	gluteal	3
2	65	M	ant. tibialis	1
3	42	F	sternohyoid	0
4	37	M	abd. rectus	2
5	26	F	flex. digitorum	7

Early transformational changes of myonuclei to presumptive myoblasts were already evident in control specimens (Fig. 10*B*). In the specimen shown, cytoplasmic morphology immediately around one pole of the myonucleus is electron dense and distinctly different from the parent sarcoplasm. There was no clear separation between this young cytoplasm and the parent cytoplasm. An electron dense body is also found in the vicinity of this transforming myonucleus. Microfilaments were apparent within the young cytoplasm.

4.4.2 Days 1, 2 and 3 after incubation - human explants

The development process seemed slow in the human explants when compared with the very rapid morphological changes observed during incubation for the hamster explants. Apart from different levels of

degeneration, there were no gross differences in the regeneration pictures of the explants between the 1st and the 3rd day of incubation. Therefore the regenerative events for these days are given as a group.

In comparison with incubated hamster muscle explants, the human explants showed greater signs of fragility and more instances of retraction. Most of the myonuclei observed had dense peripheral and central heterochromatin. Myonuclei of the human muscle seemed to exhibit a more elongated morphology with a greater incidence of membrane indentations. The number of degenerate myonuclei varied between 2 to 5% for the different muscle types. The degenerate myonuclei in the human muscle exhibited similar morphology to those seen in hamster muscle since in some, the nuclear membranes were disrupted whilst others displayed a shrunken nuclear envelope with electron dense condensed chromatin material.

Invaginated myonuclei were a common feature of the incubated explants (Figs. 8**A** to 8***C***). The walls of the invaginations were electron dense similar to that seen in hamster explants. Active imbibition of cytoplasmic elements by these invaginated myonuclei were clearly evident (Figs. 8***B*** and 8***C***). Microvesicles were again apparent in the area of imbibition (Fig. 8***B***).

As incubation progressed, the number of myonuclei declined whilst the number of presumptive myoblasts increased. Characteristic satellite cells were not observed after incubation. Myonuclear transformation to presumptive myoblasts were still evident (Fig. 11***A***). In the early transformations of myonuclei to presumptive myoblasts, the new cytoplasmic formations were always observed at one pole of the myonucleus. Incomplete membrane separations between the cytoplasm of the developing cell (Fig. 11***A***) and the sarcoplasm of the parent myofibre

provide evidence of the derivation of presumptive myoblasts from myonuclei. Again, an electron dense body is found close to the transforming myonucleus. Some of the presumptive myoblasts observed, have scanty cytoplasm with little or no developed organelles (Fig. 11*B*). These cells with little cytoplasm can easily be mistaken for myonuclei even under the electron microscope.

The mitochondria of the myofibres showed similar morphological changes to those observed in hamster muscle. Fusing mitochondrial aggregations with paracrystalline bars and dense osmiophillic bodies were often observed close to young presumptive myoblasts which were still attached to the parent sarcoplasm (Fig. 15*A*). Pleomorphic mitochondria, some being observed even before incubation (Fig. 15C), were present in some of the explants after incubation.

4.4.3 Days 4, 5 and 6 after incubation - human explants

There was no apparent variation observed in the explants for each of these days except that there was a steady increase in the number of cell forms from day to day with myotubes making their appearance on the later days. There were fewer myonuclei at this time. Degenerate ghosts of myonuclei with very little nuclear material within shrunken nuclear membrane outlines were still evident in the myofibres. Incidently, most of the degenerate myonuclei observed in both the hamster and human myofibres were located mainly in the periphery of the muscle explants. The damage and death of these myonuclei can be attributed to physical shock sustained during the preparation of the explants.

Evidence of transformational changes of myonuclei still prevailed during this period. The presence of plasmalemmal fragments between the cytoplasmic membrane of the myoblast and the basement membrane of the parent myofibre (Figs 13*A* and 13*C*) was further evidence of the origin of the presumptive myoblasts from within the parent myofibre.

Presumptive myoblasts showed greater cytoplasmic content. The cytoplasm of these cells were more differentiated with greater number of mitochondria and endoplasmic reticulum. Microtubules and microfilaments were commonly seen in the cytoplasm. Microvesicles which may be the forerunners of sarcoplasmic tubules were often observed on the peripheral margins of some myoblast especially on the side adjacent to the basement membrane (Figs. 13*B* and 15*A*).

The nuclei of these sublaminar cells displayed variable morphology. Some of the nuclei had dense peripheral heterochromatin, some had dense scattered or speckled chromatin while others had dense granular nuclei.

Sublaminal myoblasts adjacent to each other began showing signs of fusion (Fig. 13*C*). This lead to the increase in the number of mutinucleate myotubes. The myotubes also had microfilaments and microtubules in their cytoplasm. Early myofibrillar development was also observed in many of these multinucleate myotubes.

There was a gradual mitochondrial proliferation. Some early aggregations of mitochondria were observed in the subsarcolemmal regions (Fig. 15*B*). Electron dense bodies, similar to those found in the vicinity of activated myonuclei, were observed amidst the mitochondria found in clusters. The

incidence of mitochondrial clusters were not as prolific as that found in the hamster muscle at the same time of incubation.

A number of presumptive myoblasts, myoblasts and young myotubes displayed phagocytic morphology. Some of the mononucleated cells with lysosomes, extensive endoplasmic reticulum, autophagic vacuoles or phagosomes and the presence of cytoplasmic projections made identification of these cells difficult although they lay below the basement membrane (Fig. 19*A*). Myotubes which displayed some of these morphological characteristics were easier to identify by the presence of developing myofibrils in their cytoplasm. The presence of developing myofibrils are clearly shown in longitudinal section (Fig. 19*B*) and in cross section (Fig. 19*C*) of myotubes displaying some of the phagocytic characteristics.

4.4.4 Days 7 to 10 after incubation - human explants

For these days, the cell number increased with many lining up as chains in a sublaminar position (Fig. 17*A*). Cross sections of myofibres showed cells encircling the perimeter of the myofibre (Fig. 17*B*). Many clusters of cells were found capping the ends of the parent myofibres. Fusion between these cells also occurred (Fig. 17*C*).

Mitochondrial aggregations or clusters, found under convoluted basement membranes of myofibres, were observed with greater frequency. Mitochondrial aggregations also appeared at the distal ends of some myofibres. Some nuclei of presumptive myoblasts and myotubes appeared to have originated in the midst of mitochondrial aggregations, as was observed for the hamster muscle, but this was not frequent. Multinucleate

myotubes with bizarre forms of nuclei as observed in the case of hamster myotubes were not seen. Perhaps the culture duration of 10 days for the human specimens was too short in order to reveal these features.

Many myotubes showed morphological characteristics of phagocytes. The presence of young developing myofibrils in the cytoplasm of many of these myotubes confirmed their myogenic status.

4.4.4.1 "New generation cytoplasm, nuclei and cells"

Although the regenerative processes were much slower in the human explants when compared to the hamster explants, it was apparent that regeneration had already prevailed in the human muscle. Therefore, in some explants, cluster of mitochondria were observed as early as 4 days of incubation. Basement membranes of some fibres were convoluted similar to that observed in hamster explants. Organelle replacement by cytoplasmic forms were evident under these convolutions in some of the fibres (Fig. 32*A*) which had marked degenerate morphology. In this same electron micrograph, partial nuclear membrane coverage of material which may represent future nucleoplasm is evident and, perhaps, herald, the "new generation nucleus" as seen in the case of the hamster explants.

Another partially formed nucleus amidst sarcoplasmic organelles (Fig. 32*B*), taken at higher magnification, shows clearly the double membrane structure of a nucleus. Many inclusions are present within the future nucleoplasmic area. Late in the incubation period, myotubes with irregular nuclear formation (Fig. 32*C*) were observed at the distal ends of some fibres.

The cytoplasm contained numerous vacuoles and degenerate looking cytoplasmic organelles, suggesting possible derivation of this myotube as a "new generation cell".

Young cells with electron dense nuclei and dense granular cytoplasm were observed at the end of the incubation period. The cytoplasm of these cells did not contain any organelles (Fig. 32D). Furthermore, the nucleus of these "new generation cells" contained large electron dense nuclear inclusions. Close inspection of the nucleus in Fig. 32D reveals that the nuclear membrane is incomplete and terminates, at the break point, attached to small vesicles.

Mutinucleate myotubes with bizarre formations of nuclei forming caps around the distal end of myofibres and along the side of the myofibre were not observed.

4.4.5 Myonucleation - possible amitotic division

Nuclear morphology indicating possible amitotic division were observed in the controls and the incubated specimens. Therefore this issue is presented separately.

Some myoblast with developing myofibrils in their cytoplasm were seen in control specimens suggesting that the muscle used in the experiment already had regeneration features. In one such myoblast, the nucleus presents electron dense chromatin material leading from a cleft in the nucleus and branching into two columns in different directions (Fig. 21*A*). Similar pictures were observed in hamster explants. These columns perhaps

mark zones of future breaks in the nucleus. Bilobed nuclei with thin strands of nuclear material linking the lobes were often observed.

An apparently bilobed nucleus (Fig. 21*B*) linked by nuclear material, on careful examination reveals a break between the lobe and the nuclear band linking it. The morphological outline presented by this nucleus predicts 3 possible nuclei in the cell, in the future. Nuclei of other myoblasts (Fig. 22*A*) showed close invaginations which run deep into the nucleus marking lines of breaks in the nucleus.

Binucleate myotubes were also seen (Fig. 21*C*). The 2 nuclei within the myoblast have 2 little nuclear projections in each of them, on opposed sides. These little nuclear projections provide morphological evidence that these nuclei were previously linked to each other. Although it may be argued that the nuclear lobes may be linked at another level from the plane of sectioning, it is hardly likely on account of the nuclear projections displaying distinct nuclear membrane which, invariably, would be absent in tangential sections.

In Fig. 22*B*, a myotube with arrays of developing myofibrils in cross section are seen. The myotube has a bilobed nucleus linked by a ribbon of nuclear material and another nucleus that is unattached. Higher magnification of the same myoblast (Fig. 22*C*) reveals a nuclear tail or projection standing free in the cytoplasm. This nuclear projection perhaps presents the possible evidence that the other nucleus was attached at this point.

An electron micrograph of a multinucleate myotube, formed in the interstitial space in between the myofibres, is presented (Fig. 21*D*). The structure of

this myotube is similar to those produced by fusion of myoblasts in single mononucleated cell cultures. Close examination of the multinuclei reveals two nuclei attached to each other, leading evidence of amitotic division within myotubes.

Mitotic figures were not observed in the sublaminar cells.

4.5 CONCLUSION

The electron micrograph records reveal similar progression of regenerative events in both the hamster and human muscle explants. The main difference was that the regeneration process appeared to be much slower in the human explants. This seems the obvious reason for not being able to observe the prolific presentation of multinucleate myotubes with bizarre nuclei in the human muscle explants.

CHAPTER 5

DISCUSSION

5.1 INTRODUCTION

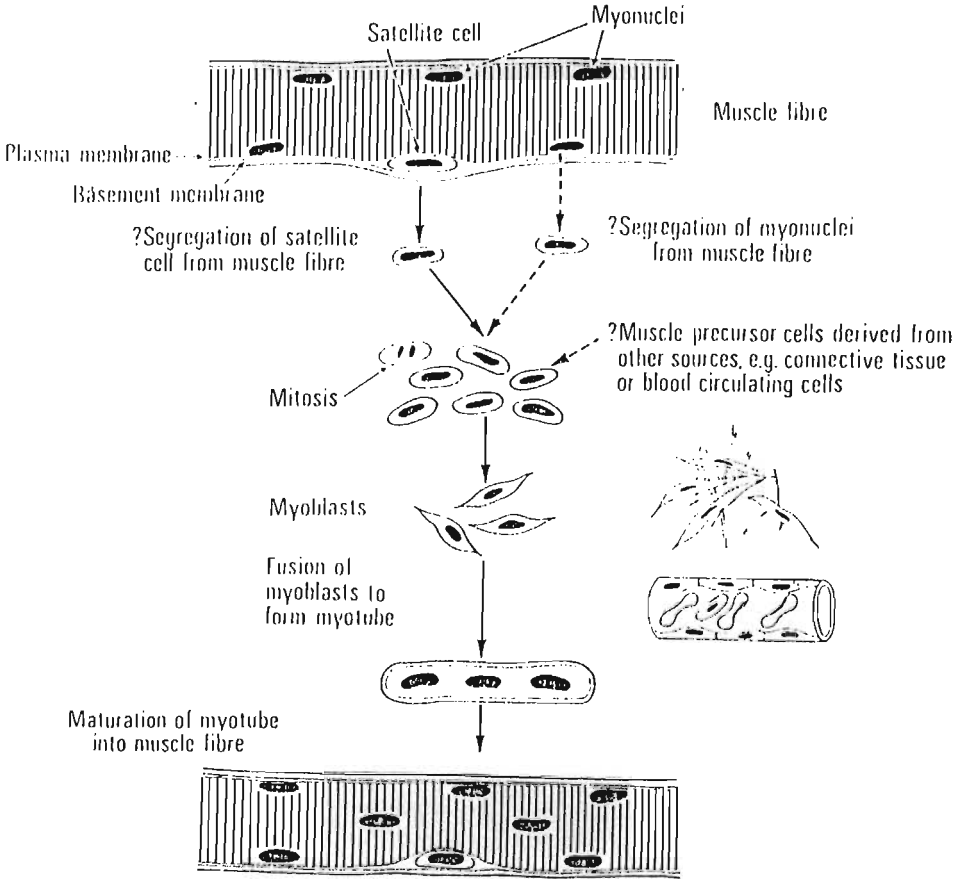
Since the discovery of the satellite cell (Mauro, 1961), most regeneration research on skeletal muscle focused on this cell with a certain amount of bias. The current consensus is that the satellite cells, remnants of embryonic myogenesis, are the precursor or myoblastic cells responsible for the initiation of regeneration in muscle whenever the need arises. Although it has not been conclusively proven that satellite cells were the precursor cells of muscle regeneration, research on regeneration has declined in recent times (Okada, 1994).

According to Okada (1994), *"There is little doubt that regeneration is one of the key, and basic mechanisms, for maintaining life, as has been clearly stated by the American biologist J. Goss. 'If there was no regeneration, there would be no life. If everything is regenerated, there would be no death'. Thus, studies on regeneration are part of the essential scientific basis to discussion of that most important philosophical problem of 'life and death'"*.

The importance and the need for regeneration studies on skeletal muscle can not be over emphasized, especially in view of the fact that whatever therapy becomes available in the future for muscular dystrophy, it will, out of necessity, depend on the muscle's ability to regenerate. There is, therefore, a genuine need to continue research to precisely understand the mechanism

of regeneration. Most importantly, the derivation of precursor cells that are involved in muscle regeneration must be conclusively identified because, these precursor cells are the ones that may be the targets of future therapies.

5.2 PREVAILING HYPOTHESES ON THE DERIVATION OF PRESUMPTIVE MYOBLASTS DURING MUSCLE REGENERATION



Schematic representation of the different hypotheses on the derivation of muscle precursor cells by Sloper and Partridge (1980).

The origin of the mononucleated myogenic cell has not been proven beyond doubt in any of the muscle regeneration models used (Carlson, 1973; Bischoff, 1979; Sloper and Partridge, 1980) and therefore remains a debatable issue. The different hypotheses on the derivation of precursor cells during muscle regeneration are illustrated in the diagram above by Sloper and Partridge (1980).

There are two main schools of thought existing on this issue as mentioned in Chapter 2. The one school supports the hypothesis that satellite cells are the precursor cells involved in muscle regeneration (Church et al., 1966; Moss and Leblond, 1971; Ontell, 1974/1975; Schmalbruch, 1976; Snow, 1977a&b/1979; Campion 1984), and this is the current consensus. The other school believes that mononucleated precursor cells of muscle regeneration are derived from myonuclei (Hay, 1959/1979; Lee, 1965; Reznik, 1969/1976; Teravainen, 1970; Hess and Rosner, 1970). According to this school, myonuclei were capable of undergoing dedifferentiation to form new cells if and when muscle regeneration was required.

There were also some suggestions that connective tissue cells, such as fibroblasts and macrophages, could also be involved in the contribution of new cells during regeneration (Sloper and Partridge, 1980).

5.3 EARLIER METHODS OF REGENERATION STUDY

Muscle regeneration studies began over 135 years ago (Bottcher, 1858; Waldeyer, 1865 - cited in Campion, 1984). Since then, studies in regeneration were performed in many different experimental situations.

These are itemised with references under literature review. Just to reiterate briefly, various techniques were tried and tested to induce regeneration in living animals and under culture conditions in the laboratory. Speidel as early as 1938 used tadpoles to study regeneration. He provided clues for the progressive changes which took place from single spindle shaped myoblast up to the formation of myofibres. These findings held true for regeneration in both the animal and human muscle in subsequent studies that followed, and this also applies to the current study (Figs. 1 & 2).

The study methods may be classified into two major groups. Group one comprised the study of regeneration by microscopical comparisons of muscle from foetuses, the young and the adult human, as well as from many animal species (Ishikawa, 1966; Shafiq et al., 1967). Muscle autografts (Schmalbruch, 1977; Carlson et al., 1979; Lipton and Schultz, 1979), muscle transplants (Jasmin and Bokdawala, 1970; Cosmos et al., 1979), minced muscle implants (Mong, 1977; Snow, 1977a&b/1978/1979), denervated muscle (Campion, 1978; Kelly, 1979) and muscle injured in a variety of ways (Allbrook et al., 1965; Shafiq and Gorycki, 1965; Mazanet et al., 1982) were employed to pursue the development and regeneration of muscle.

Group two employed tissue culture. Essentially, there were two culture techniques used. One of which employed single explants in clot cultures (Geiger and Garvin, 1957; Skeate et al., 1969; Bishop et al., 1971; Dubowitz, 1973; Witkowski and Dubowitz, 1975), or free-floating multi-explants (Askanas, 1979; Askanas and Engel, 1982; Tautu and Jasmin, 1982; Jasmin et al., 1984a&b; Delaporte et al., 1984; Ecob-Prince and Brown, 1988), or single myofibres in culture (Bischoff, 1972/1975/ 1979;

Konigsberg, 1975; Konigsberg, 1979; Rubin et al., 1979). The other culture technique employed enzymatically dissociated mononucleated cells (Hauschka, 1974; Yasin et al., 1977; Thompson, 1980).

The clot culture technique has now become obsolete. Most of the culture methods, unfortunately, were not used to identify the precursor cells of regeneration. Instead, they concentrated on the growth and developmental characteristics of the cells that sprouted from the explants or those grown from mononucleated cells liberated by enzymic dissociation of muscle. Cultures of diseased muscle were used in the hope that the structural changes and biochemical defects, observed in biopsies, would be reincarnated, ***in vitro***, and this would then enable attempts at treatment.

Apart from observing the growth characteristics during regeneration of muscle, all the earlier work, engaging any of the techniques mentioned, had one thing in common, and that was to test the various hypotheses that existed on the pathogenesis of muscular dystrophy. In general, it may be summarised that the growth characteristics of diseased and normal muscle did not indicate any appreciable growth and morphological differences in culture (Dubowitz, 1973).

With the identification of the gene defect and the gene product, dystrophin, many of the conflicting findings, such as cluster formation in cultures of DMD muscle (Walsh et al., 1981; Thompson et al., 1983) and a neurogenic (Dubowitz, 1973) or ischaemic (Vracko and Benditt, 1972; Lipton, 1979) cause of the defect in muscular dystrophy, have become redundant and held only historic and academic merit.

Nevertheless, the culture techniques used are not without merit and will be in continuous use in the future. In recent times, the techniques have been perfected to isolate and harvest massive numbers of muscle cell clones (Konigsberg, 1963; Hauschka, 1974; Yasin et al., 1982). The cell clones are used in myoblast transplant and gene transfer therapy research. The cultured cells are also useful for the identification of the different dystrophies, including proper prenatal diagnosis and establishing carrier status in patients.

5.4 ADVANTAGES OF THE EXPLANT CULTURES IN THE USE OF MUSCLE REGENERATION STUDY

It is well documented (Askanas, 1979; Tautu and Jasmin, 1982; Jasmin et al., 1984a) that skeletal muscle explants in culture sprout young myoblasts and, therefore, must exhibit regenerative changes. Askanas (1979) considered regeneration of diseased human muscle in culture a valuable tool to study the pathogenesis of neuromuscular diseases, and that it presented a new frontier in the study of the disorders. The explant technique, nowadays, have come a long way since the early clot culture technique. Minced muscle grown as free floating explants in aqueous unclotted culture media provided better migration and growth of presumptive myoblasts (Askanas and Engel, 1975; Tautu and Jasmin, 1982; Jasmin et al., 1984).

Whilst most of the previous studies used cultured explants and single myofibres to evaluate the growth and development of the young cells sprouting (Fig. 2B) out from them, cultured explants were used in the current study to monitor the regenerative changes that occurred within them

for 10 consecutive days after incubation. Muscle explants in culture provide an ideal model and afford an excellent opportunity to study early regenerative changes on a day to day basis, on account of the easy accessibility and availability of the explants from animals or biopsies from patients. Such study, otherwise, is difficult in an intact animal that would have to be subjected to repeated surgical procedures that would be painful, both for the animals and the researcher.

Regeneration studies, performed on injured muscle by autografts, transplants and minced muscle implants, were not successful in establishing conclusively the origin of precursor cells. This was largely due to the formation of blood clots, poor perfusion, necrotic debris and migrating connective tissue cells such as phagocytes and fibroblasts in the experimental muscle in, **in vivo** regeneration studies (Allbrook, 1962; Snow, 1979; Baker and Poindextor, 1991). All these conditions made the detection of early changes of regeneration within the experimental muscle difficult. Ultrastructural investigations in incubated human muscle explants in Maximow double coverslip chambers on collagen-coated plastic coverslips with explants fed 1 drop of growth medium 3 times a week were beset with similar problems (Mendell et al., 1972). Single myofibres grown in culture resulted in all the myonuclei disappearing (Bischoff, 1979).

On the other hand, the muscle explants, being grown in a controlled laboratory environment, were free of other " outside-muscle" influences (Askanas, 1979). The explant cultures (Fig. 2) were clean without debris, had good perfusion of nutrients, were free of blood clots and suffered minimal migrating or invading cells. Thus, in the present study, the early tracing and recording of events involved in muscle regeneration were made possible and more meaningful.

5.5 MORPHOLOGICAL CHANGES OBSERVED IN BOTH HAMSTER AND HUMAN EXPLANTS FOR 10 CONSECUTIVE DAYS OF INCUBATION

Hamster muscle explants were prepared from the thigh muscle of apparently healthy adult male and female hamsters. Human muscle, on the other hand, was obtained from patients undergoing surgery for conditions other than muscle diseases. There was no alternative but to use whatever muscle was available at the hospital surgical theatre. Therefore, human muscle obtained from different sites in the body were used in this study. However, the advantage of having obtained muscle from different sites was that it helped to observe that the regenerative changes held true for the all of them. Furthermore, regenerative changes were similar in the muscle from either sex, both in the hamster and in the human.

The present study focused attention on the behaviour of myonuclei, origin of myoblasts and the role of phagocytes during regeneration in hamster and human skeletal muscle explants. Other morphological changes observed are also discussed.

5.5.1 The status of myofibres of the cultured explants

A problem encountered in the use of explants was that after incubation they were extremely delicate and fragile and, therefore, easily susceptible to disruption of the structural integrity if not handled with care. Unlike most other regeneration studies (Bischoff, 1979; Reznik, 1969) viable myonuclei prevailed for several days of incubation, although there were a small percentage of degenerate myonuclei. The degeneration of these myonuclei

were apparently due to mechanical damage and were observed mainly in the peripheral myofibres of the explants. The ultrastructural integrity of the muscle fibres in the control pre-incubation or uncultured explants appeared normal, taking into consideration the mechanical injury sustained by the explants during their preparation for culture.

On incubation, the human muscle seemed more susceptible to retraction than hamster muscle in the first few days, and normalised thereafter. As incubation proceeded, myofibres in the explants began to exhibit oedema, fibrillar disorganisation and mitochondrial degeneration and regeneration. All of these changes were also reported in other studies concerning muscle regeneration (Allbrook, 1962; Reznik, 1969; Lipton, 1979; Anderson et al., 1987; Baker and Poindextor, 1991). In the midst of these seemingly degenerate myofibres, the regenerative processes were set in motion. The sarcoplasmic morphology of the explants will be discussed in more detail later on in this chapter.

5.5.2 EM identification of the origin of myoblasts

It is appropriate at this stage to again define satellite cells, myoblasts and myotubes. Satellite cells, considered to be a remnant of embryonic myogenesis, first observed in frog skeletal muscle (Mauro, 1961), were uninucleate cells having dense peripheral heterochromatin and scanty cytoplasm with few organelles. They must be located between the basement membrane and the plasmalemma (sarcolemma) of the muscle fibre. Definitions for myoblasts varied (Allbrook, 1962; Carlson, 1973), but they were generally accepted as precursors of muscle fibres. Those cells whose destiny was presumed to be future myoblasts were commonly

referred to as presumptive myoblasts. Myotubes were either mononucleate cells or multinucleate syncytia, with immature myofibrils, which go on to develop into young myofibres.

In a preliminary work, EM study of cultured mononucleated cells embedded, ***in situ***, in the culture flasks, showed characteristic myoblasts with well developed organelles (Fig 1). The nuclei of the cells were dense granular, elongated without deep indentations of peripheral heterochromatin and not similar to that of the satellite cell first described by Mauro (1961). Some of these cells revealed early developing myofibrillar elements in the peripheral regions of the cytoplasm. These cells, with developing myofibrils, also exhibited fusion between neighbouring cells. Electron dense lysosomal-like cytoplasmic bodies were numerous in the cytoplasm of the spindle shaped cells. In the current study, similar structures were also observed proximal to transforming myonuclei and young presumptive myoblasts as though they signalled regenerative events (Figs. 6*B*, 6*D*, 10*B* and 11*A*). Pellets of mononucleated cells, harvested from the muscle cultures, exhibited morphologically round cells with fine cytoplasmic projections. The important aspect of the morphology of these cells was that the nuclei did not show any irregular or bizarre outlines, and they seemed to have either one or two prominent nucleoli (Fig. 1).

Light microscopy of live cell cultures revealed that the cultures were free of debris and that many of the cells that sprouted out of the explants were confirmed myoblasts because they fused and formed multinucleate myotubes (Fig. 2). This implied that the culture environment was adequate and promoted growth in the muscle explants. Visual comparison of light micrographs between uncultured control explants and cultured explants,

revealed an obvious increase in the number of myofibre nuclei (Figs. 3 and 4) as the incubation time increased. Whilst some of the nuclei clearly belonged to cell forms, it would be erroneous to regard the balance of nuclei as myonuclei, because the limited resolution of the light microscope made it difficult to precisely delineate a myonucleus from a mononucleate cell which had scanty basophilic cytoplasm.

Definite signs of regeneration were evident in light micrographs of cultured explants 3 days after incubation from hamster (Figs. 3*C* to 3*F*) and 5 days after incubation from human muscles (Figs. 4*D* to 4*F*), by the appearance of seemingly multinucleate, basophilic cell forms resembling myotubes. Similar findings were recorded by others (Lash et al., 1957; Reznik, 1969; Carpenter, 1990; Baker and Poindextor, 1991). Again, it was difficult with the light microscope to positively identify whether the nuclei were a part of the myotubes, or whether they were mononucleate cells adjacent to each other, and therefore appearing as though they were multinucleate myotubes.

5.5.2.1 Derivation of presumptive myoblasts from myonuclei

An intense electron microscopic investigation of the muscle explants in the present study helped to clarify and identify the ultrastructural changes which occurred early in the regenerative process. However, some early regenerative features such as myonuclear transformations were already present in the control muscle samples obtained from the patients. This could be attributed to the fact that some of the samples came from sites of injury. In addition, the effects of drugs and anaesthetics used on the patients could also have contributed to the regenerative features. Nevertheless, this did not affect the aims of the study.

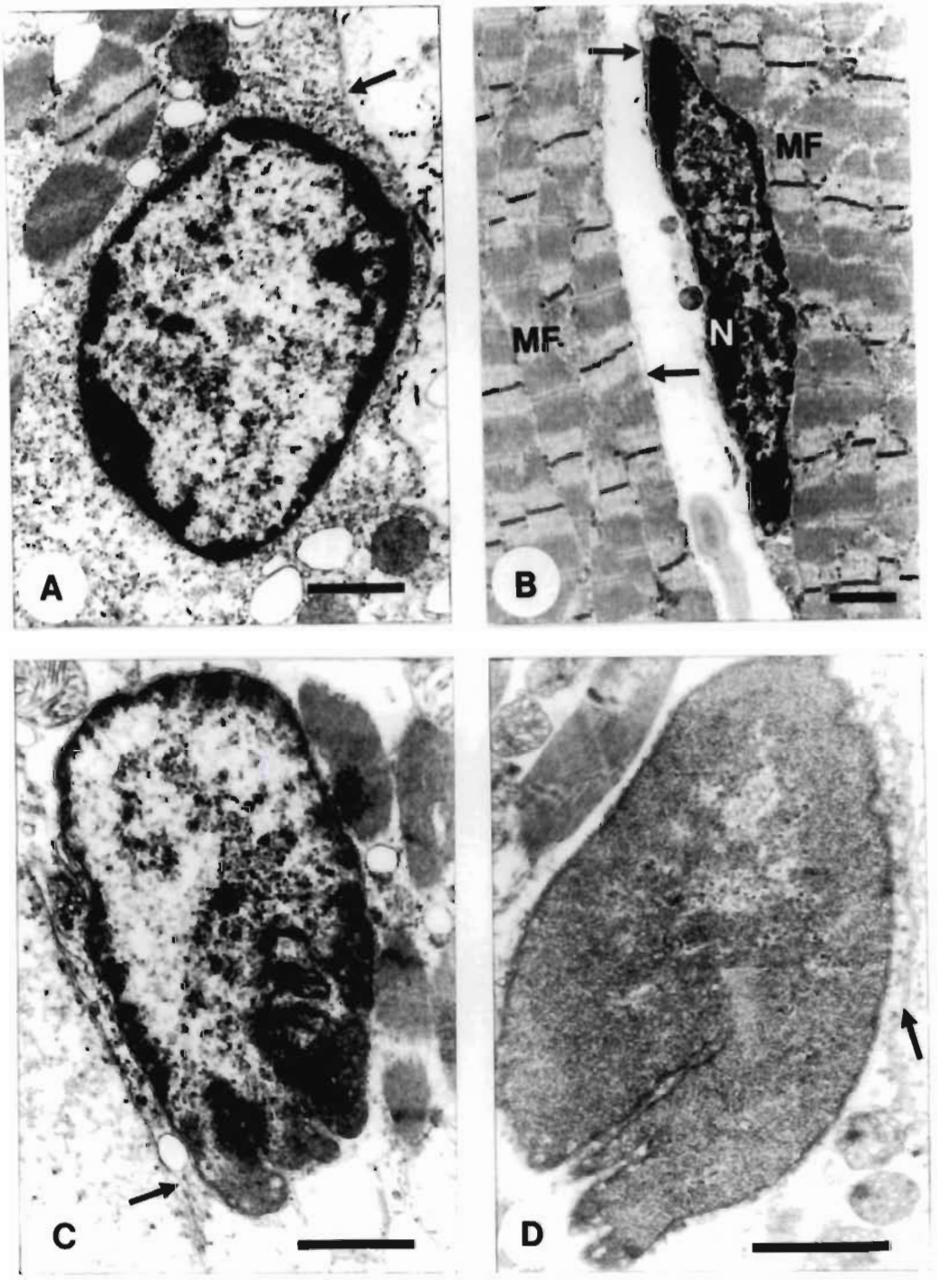


Figure 5. Electron micrographs *A*, *B*, *C* & *D* represent some of the myonuclear morphology observed in hamster explants up to 5 days of incubation including controls. *A:* Typical euchromatic myonucleus mainly of controls. *B:* Dense peripheral heterochromatin in myonuclei, in controls and day 1 incubation, resembling satellite cell nucleus. *C* & *D:* Myonuclei display invaginations. *C*, with electron dense material adjacent to invaginations was probably a stage in the transformation to *D* with dense homogeneous granular material. N, myonucleus; MF, myofibre; arrows, basement membrane. Bar = 1 μm.

5.5.2.2 Morphological transformations of myonuclei

Typical euchromatic myonuclei (Dubowitz, 1985) were observed mainly in the control specimens (Figs. 5*A* and 6*A*) but were rare in the incubated samples. Most of the myonuclei of the human control and incubated explants displayed variable morphology. There were myonuclei with dense peripheral chromatin (Fig. 6*B* and see appendix I & II), typical of nuclei of satellite cells (Mauro, 1961). When seen in longitudinal sections (Fig. 5*B*) of intact myofibres, these myonuclei could easily be mistaken for satellite cells.

Dense granular myonuclei, some of them with invaginations of their membranes (Fig. 5*D*), were a common feature only in the incubated hamster explants. Similar dense granular myonuclei, some with a prominent nucleolus (Fig. 6*D*), were also found in control and incubated explants of human muscle. The presence of these dense granular myonuclei in the control human muscle samples was a clear sign that the human muscle had regenerative features even before incubation.

The dense granular myonuclei observed in the explants closely resembled the nuclei seen in the mononucleated myoblasts from the culture flasks. These dense granular myonuclei were also observed in other regeneration studies (Lash et al., 1957; Allbrook, 1962), and these were possibly identified as pyknotic nuclei (Reznik, 1969; Witkowski, 1977; Snow, 1979). Furthermore, in the newt limb muscle, the euchromatic myonuclei become heterochromatic during the process of dedifferentiation (Hay, 1979).

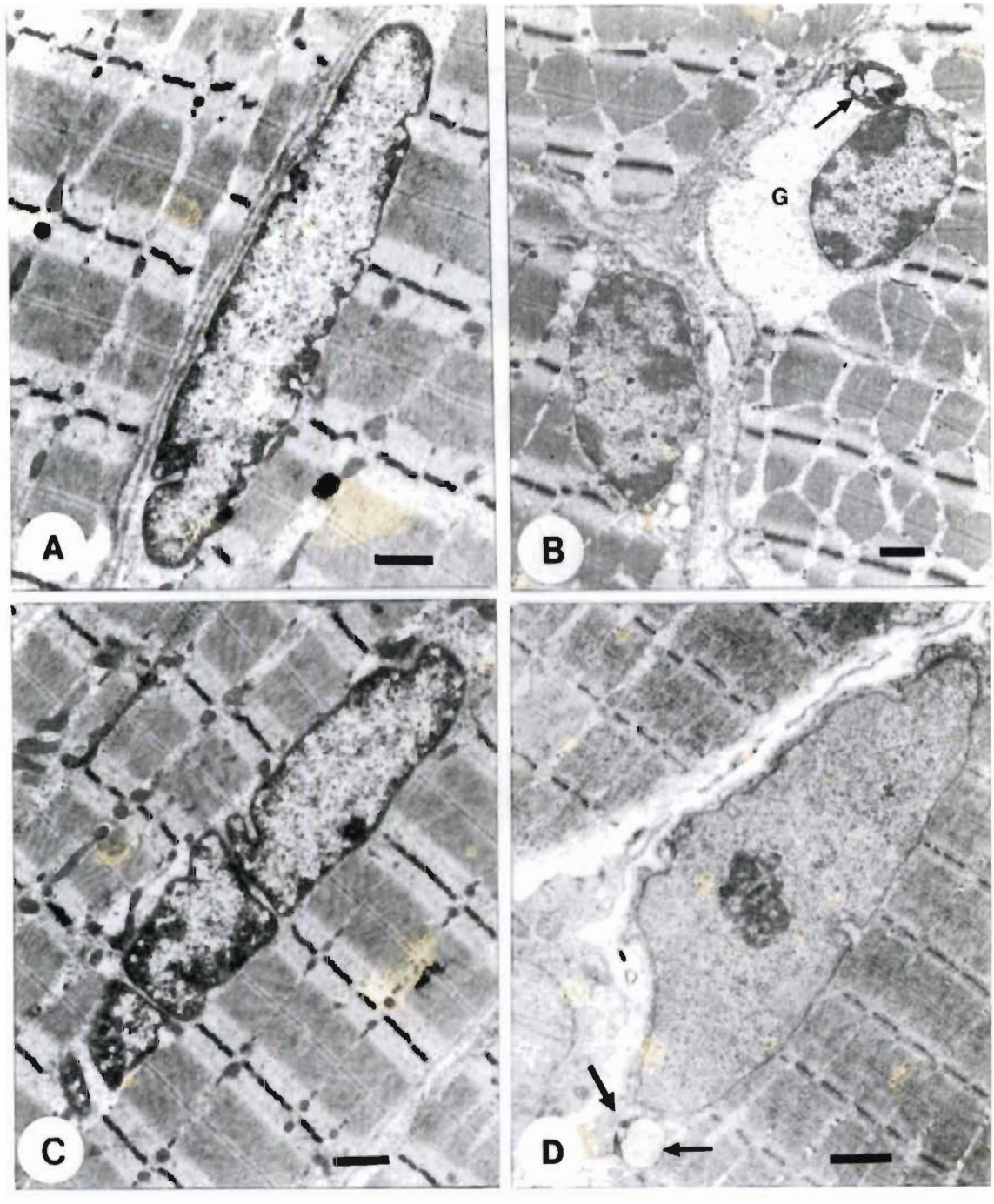


Figure 6. Electron micrographs of myonuclei observed in human control muscle explants before culture. *A:* Normal myonucleus, slightly heterochromatic. *B:* Heterochromatic myonuclei, one having what appears like a nuclear bud, is surrounded by glycogen. *C:* Centrally located beaded nuclei commonly seen in the human explants. *D:* An activated myonucleus with granular nucleoplasm and a prominent nucleolus. There is speculation that the slightly electron dense cytoplasm (thick arrow) probably is young cytoplasm marking the beginning of a presumptive myoblast from the myonucleus. The electron dense bodies with vacuolations (thin arrows) near the myonucleus in *B* and *D* were commonly seen in proximity to myonuclei that showed signs of transformation to a cell. Bar = 1 μm.

Myonuclei with deep indentations (Fig. 7*B*) and beaded myonuclei, some centrally located in the myofibre (Fig. 6*C*), were often seen in the human muscle explants. These myonuclei with indentations and bead formations were also shown in dystrophic muscle and DMD carriers (Sewry et al., 1985). High magnification of some of the indented myonuclei (Fig. 7*B*) revealed constrictions and double lines which appeared to mark possible lines of segmentation of the myonucleus giving a clue perhaps to possible amitotic division of the myonucleus. Myonuclei appearing close to each other in bead formation with each segment having a prominent nucleolus (Fig. 7*A*) were probably formed by such segmentation. It may be argued that these myonuclei in bead formation were parts of a single myonucleus with indentations, and being sectioned along the indentations, gave the appearance that they were independent nuclei. That argument may be true, but the fact that 3 clear nucleoli are present along the segments, one in each, gives an indication that they were independent nuclei. Nuclei of myoblasts in cultures commonly revealed either one or two nucleoli. If myonuclei were derived by the fusion of myoblasts during myogenesis, then it would be rare for a myonucleus to have 3 nucleoli. The segmentation lines seen in the highly indented myonuclei also indicated the possibility that these separate segments may have arisen by amitotic division.

Deep invaginations of membranes in semi euchromatic myonuclei were a common observation in incubated explants of both hamster and human muscle explants. The electron dense material was invariably present in the nucleoplasm adjacent to the walls of the invaginations (Figs. 5*C*, 8*A* and 8*C*). These myonuclei seemed as though they were imbibing cytoplasmic elements in the area of the invaginated troughs. This would explain the electron dense material in the region of the invaginations, and would perhaps also explain the origin of the many dense granular myonuclei.

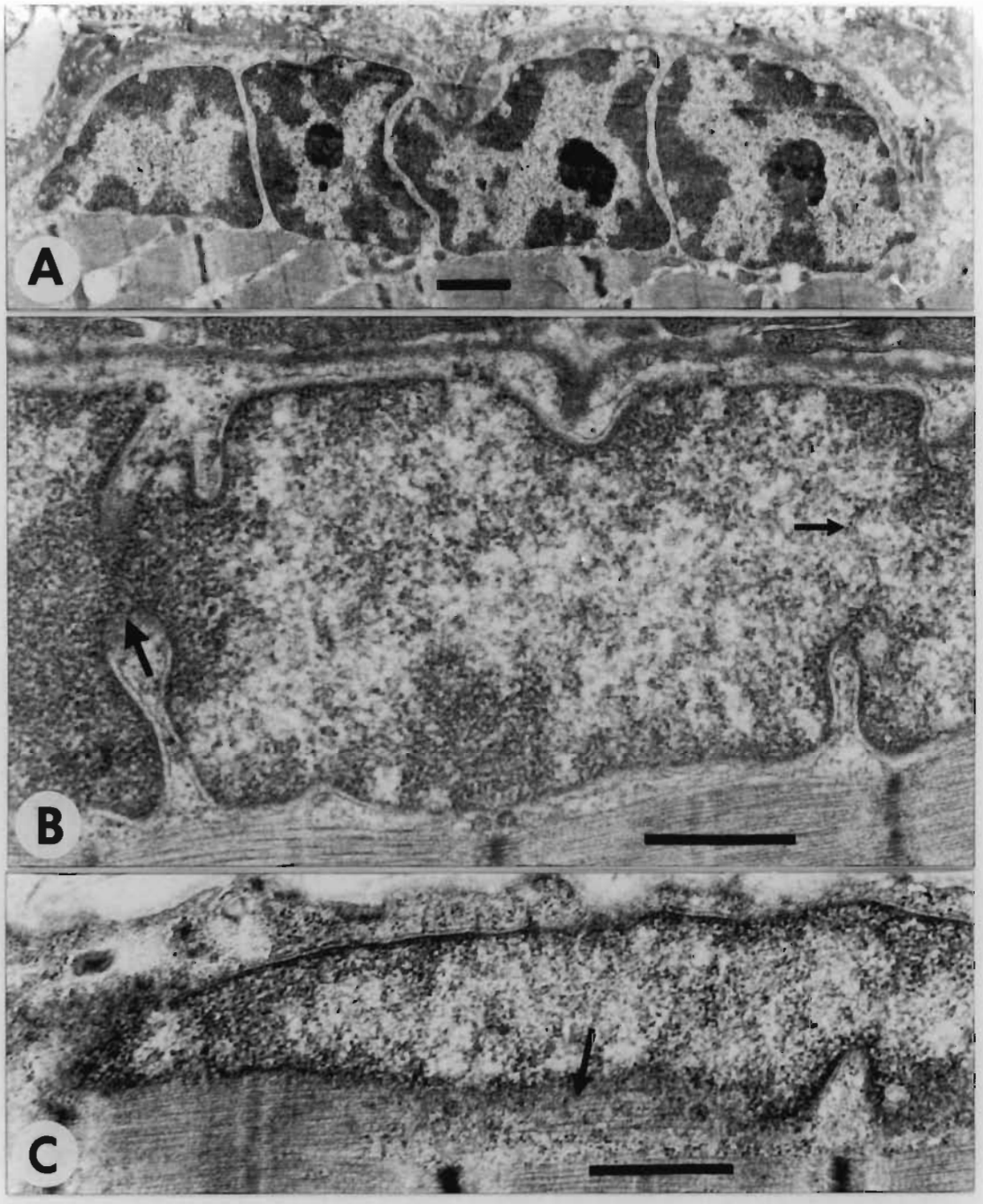


Figure 7. Electron micrographs of nuclei observed in human control explants. *A:* Beaded nuclei with a prominent nucleolus in some. *B:* Part of a myonucleus observed with constrictions (thick arrow) in an area dense with chromatin. This constriction and a faint double line (thin arrow) perhaps give a clue to the manner in which beaded nuclei are formed. *C:* Myonucleus apparently imbibing myofibrillar material (arrow) with no apparent nuclear membrane in this area of activity. The status of this myonucleus, whether it is a formed nucleus actively imbibing cytoplasmic material or a myonucleus in its formative stages from cytoplasmic material, remains a question.
Bar = 1 μm in *A* and 0,5 μm in *B* and *C*.

Deep invaginations of the nuclear membranes trapping cytoplasmic contents within the nucleus were also observed in other studies (Mastaglia and Walton, 1971; Anderson et al., 1987). Imbibition of cytoplasmic elements was clearly seen in the higher magnifications (Figs. 8*B* and 8*C*). The nuclear membranes in the area of interaction with the cytoplasmic elements were disrupted, as clearly seen in the figures presented. Figure 7*C* shows a myonucleus from a control human explant, apparently imbibing myofibrillar material with no apparent nuclear membrane along this area of activity. It is difficult to decide on the status of this myonucleus, whether it is a fully fledged myonucleus actively imbibing cytoplasmic material or whether it is a myonucleus in its formative stage from cytoplasmic material (see "new generation nuclei" discussed later in this chapter).

Previous work (Lash et al., 1957; Witkowski, 1977) also suggested that the early stages of muscle regeneration were denoted by myonuclear activity. Therefore, the dense heterochromatic and granular myonuclei observed by them and in this study, were probably euchromatic myonuclei that were transformed to a stage preceding the formation of presumptive myoblasts of myonuclear origin. It is important to note here that myoblasts in other studies were also described as having dense granular nuclei (Allbrook, 1962). The nuclei observed in the above studies closely resembled the granular myonuclei described in this study and, therefore, one might deduce that the dense granular myonuclei develop into presumptive myoblast.

The centrally placed nuclei, observed in the human control muscle, were typical of centronuclei observed in dystrophic muscle and, their presence was suggestive of various stages of regeneration (Walker, 1962; Schmalbruch, 1979), and often the central nuclei were observed to be pyknotic (Hudgeson et al., 1967).

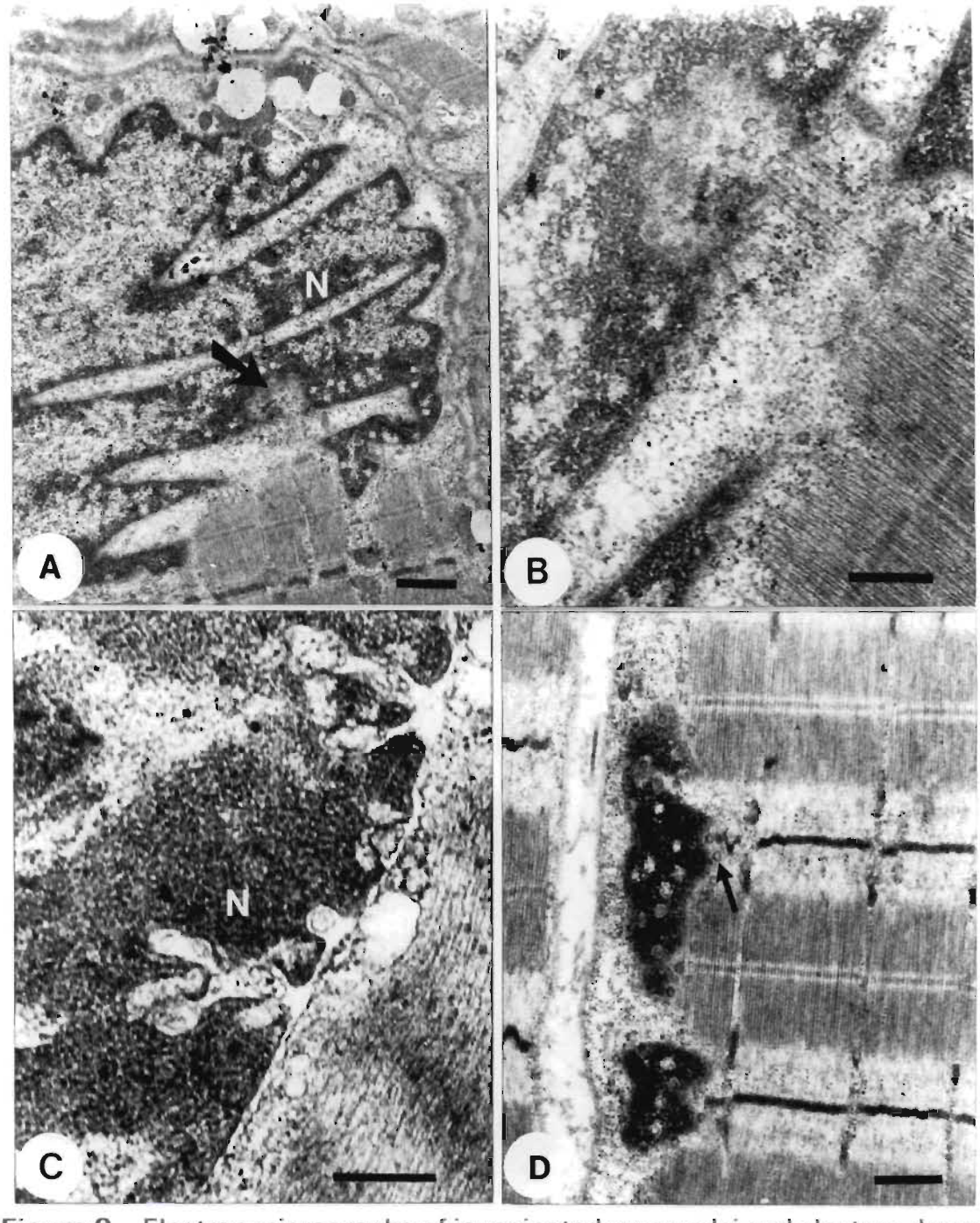


Figure 8. Electron micrographs of invaginated myonuclei and electron dense cytoplasmic bodies in human explants. *A* and *C:* Parts of invaginated myonuclei (N) commonly seen after incubation. Nuclei actively imbibing cytoplasmic elements which explain the dense heterochromatic appearance of regions adjacent to the invaginations. *A:* Imbibition of myofibrillar elements (arrow). *B:* higher magnification clearly demonstrating imbibition in *A*. *D:* Dense nuclei like bodies without apparent limiting membrane, commonly seen in controls and incubated explants. They were also observed in hamster muscle. Dense bodies, associated with microvesicles (arrow), were found opposed to and involving the Z band material.
Bar = 2 μm in *A* & *D* and 0,25 μm in *B* & *C*.

5.5.2.3 Myonuclear derivation of myoblasts

Although it is agreed that satellite cells observed in mature muscle serves the function of precursors of myoblasts, but there is no conclusive evidence, as yet, to accept that these cells were embryological remnants of myogenesis, lying in reserve. On the contrary, the present study supports the view that development of new presumptive myoblasts from myonuclei occurs whenever the need arose for regeneration of muscle. The presumptive myoblasts segregate from the sarcoplasm of the parent myofibre by newly formed membranes separating them from the parent myofibre, and therefore become placed between the plasmalemma and the basement membrane. In this manner, new 'satellite cells' are formed. There must be a continuous, slow turnover of these cells in normal muscle, which is why they are always reported to be present (Allbrook, 1971; Wakayama and Schotland, 1979; Watkins and Cullen, 1988), but in very small numbers, counts varying according to age and the physical condition of the muscle. On the other hand, if the satellite cells were true remnants of embryonic myogenesis, then there should be no decline in their number once mature muscle is established. From the literature, it becomes obvious that the numbers of satellites are closely linked to the physiological state of the muscle (Campion, 1984).

Some presumptive myoblasts (Figs. 9*D* and 12*A* and see appendix I) closely resembled satellite cells (Figs. 9*A* and 10*A*). The only difference between the satellite cells and these presumptive myoblasts was that the latter were within the myofibre, and in many cases (Figs. 9*A* and 9*D*), were still attached to the parent sarcoplasm.

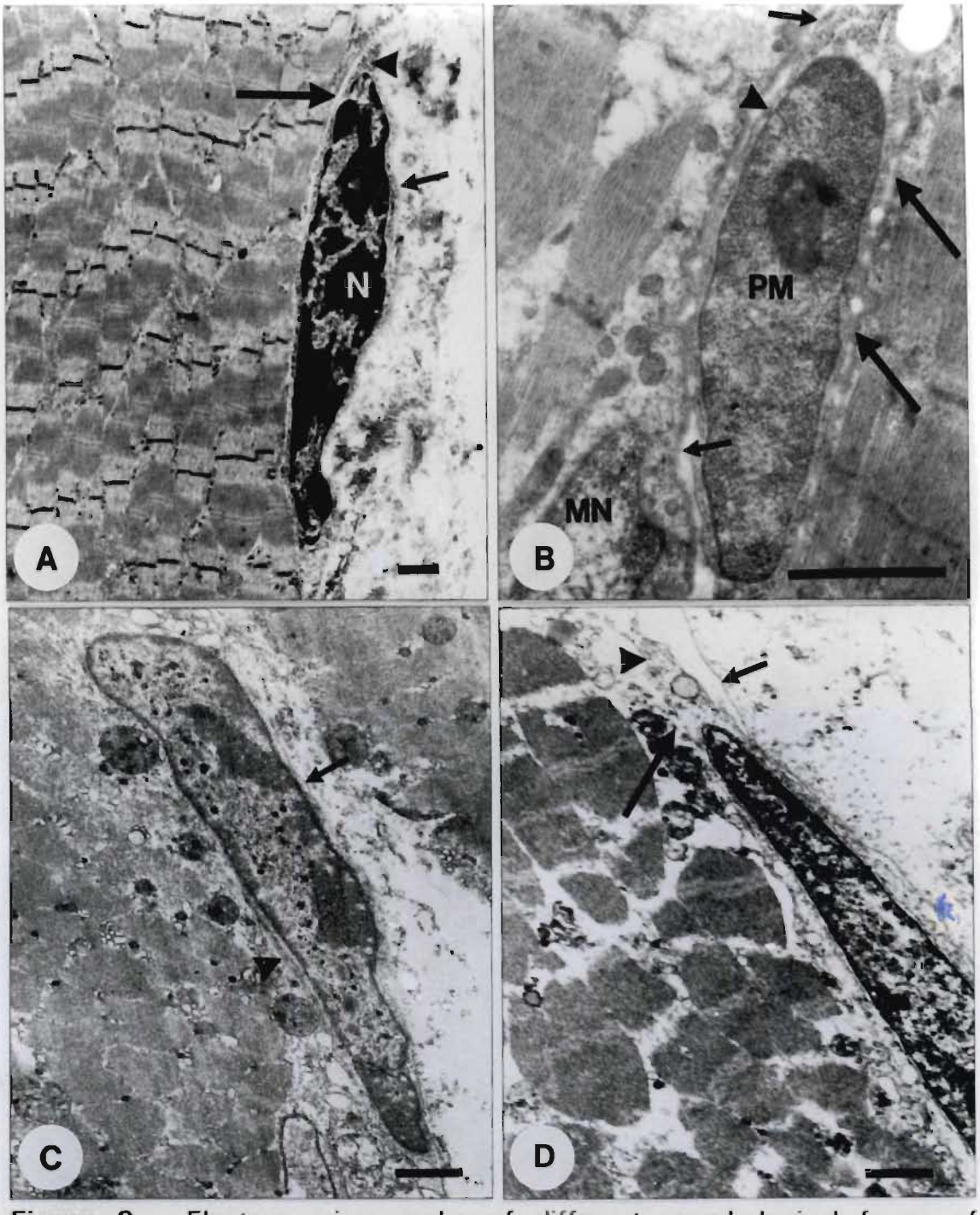


Figure 9. Electron micrographs of different morphological forms of sublaminar cells in hamster explants. *A:* Satellite cell with dense heterochromatic nucleus and scanty cytoplasm lying between the basement membrane and plasmalemma of the myofibre, in controls. *B:* Presumptive myoblasts in controls and up to 4 days incubation. Cell below basement membrane appears attached at points to the myofibre. *C:* Presumptive myoblast with scanty cytoplasm after incubation. A nuclear break is visible at one pole, this perhaps being an early myotube. *D:* Early transformation of the myonucleus to a myoblast with scanty cytoplasm at one pole of the nucleus. N, nucleus of cell forms; MN, myonucleus; PM, presumptive myoblast; short arrows, basement membrane; long arrows, plasmalemma of myofibre; arrowhead, cytoplasm of young cell forms. Bar = 1 μm.

The attachment of the presumptive myoblasts to the parent sarcoplasm implied that these cells must have their origin from within the myofibre. The morphological similarity between these two cells lends support for the view that the presumptive myoblast must be a stage in the development of satellite cells. If this is contested with the assumption that the satellite cell was undergoing fusion with the myofibre and therefore the attachment, then one would expect, at least in some cases, to see another satellite cell nearby because argument in literature is that satellite cells divide mitotically and contribute one of the cells to the myofibre (Snow, 1979).

Evidence of partial transformation of a number of myonuclei to presumptive myoblasts further confirms the derivation of muscle precursor cells from myonuclei. Figure 10*B* distinctly shows new cytoplasm formation of different electron density from that of the parent sarcoplasm around one pole of the myonucleus with no distinct membrane separating the new cytoplasm from the parent sarcoplasm. Other myonuclear transformations to a cell, where the beginning of membrane separation between the new cytoplasm formed at one pole of the nucleus and the parent sarcoplasm was observed, provide more evidence (Fig. 11*A*).

Many of the presumptive myoblasts that were attached to the parent sarcoplasm (Figs. 9*B*, 9*D*, 11*A*, 11*B* and 12*A*) had very scanty cytoplasm with little or no developed organelles, revealing their immaturity. If the argument was to be presented that these were satellite cells fused with the myofibre, then as activated satellite cells they ought to have more cytoplasm and well developed organelles.

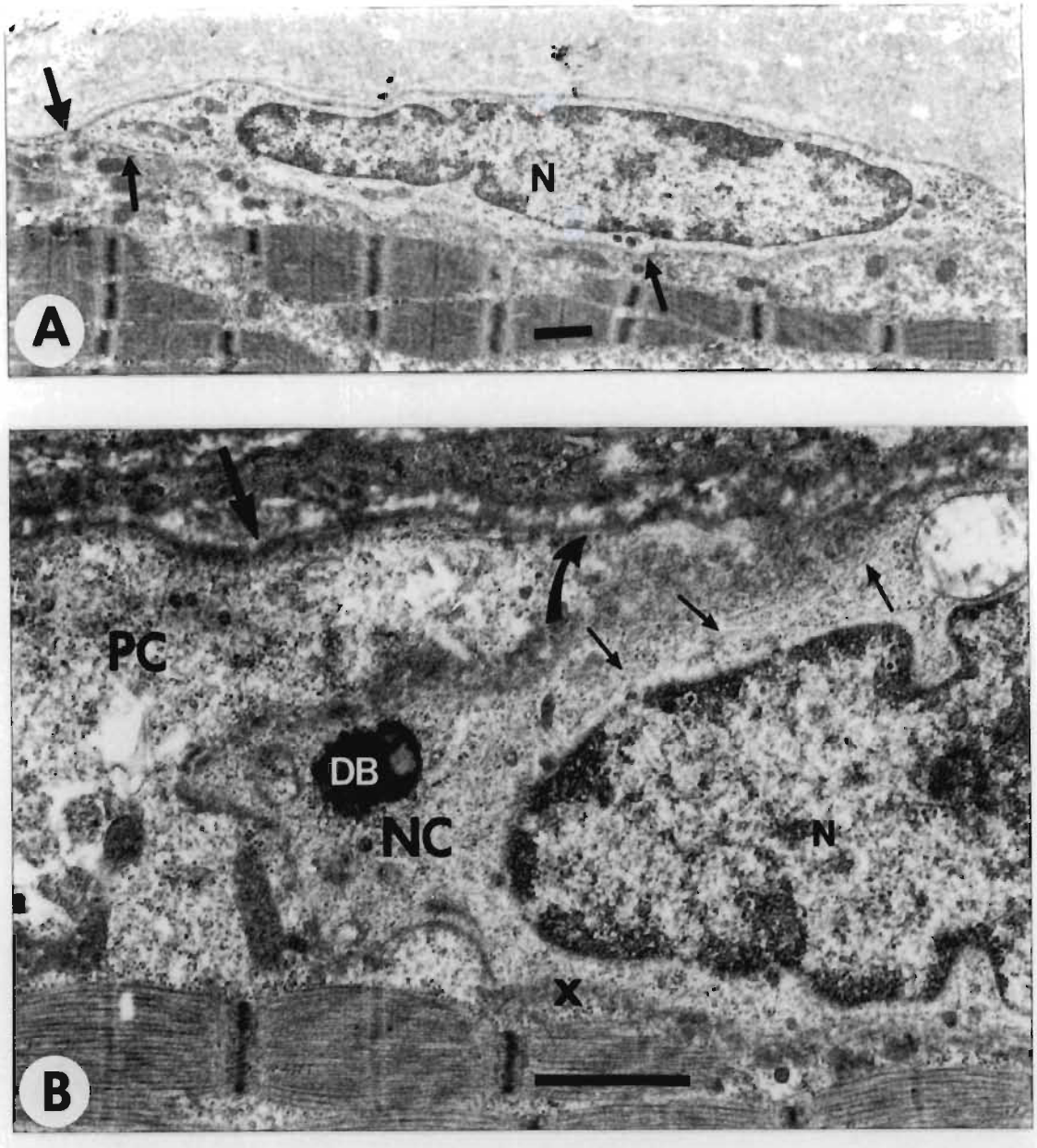


Figure 10. Electron micrographs of satellite cell and presumptive myoblast in control human explants. *A:* Typical satellite cell lying between the basement membrane (thick arrow) and plasmalemma of myofibre (thin arrow). ***B:*** Developing presumptive myoblast below the sarcolemma with development of dense new cytoplasm (NC) around part of the nucleus with no distinct membrane separating it from sarcoplasm (PC) of the parent myofibre. At point X there is no clear separation of the young cell and parent myofibre. DB, dense body is again near the nucleus. Microfilaments (small arrow) are apparent within the new cytoplasmic zone suggesting the myoblastic status. The cell lies under the basement membrane (large arrow) and the plasmalemma (curved arrow) of the parent myofibre. N, nucleus. Bar = 1 μm.

Electron dense cytoplasmic bodies with vacuolations were frequently observed proximal to activated myonuclei (Figs. 6*B* and 6*D*), in the cytoplasm of myonuclei showing partial transformations (Fig. 10*B*) and in the vicinity of other transforming myonuclei (Fig. 11*B*).

Similar electron dense structures were described as lipofuscin observed in DMD carrier muscle (Sewry, 1985) and as residual body (Anderson et al., 1987) in mdx mouse muscle. These lysosome-like dense bodies were also demonstrated in the preclinical stages of dystrophy (Hudgeson, 1967), in regenerating myotubes in dystrophic foci (Lipton, 1979) and in proximity to the nuclei of young fibres (Mastaglia and Walton, 1971). Such electron dense bodies were numerous in cultured mononucleated myoblasts (Fig. 1) appearing as dense lysosomes. Whatever they may be, they seemed to signal regeneration or growth.

Some presumptive myoblasts had nuclei with scattered dense chromatin (Figs. 9*C* and 9*D*) and others had nuclei with diffuse granular nucleoplasm (Figs. 9*B* and 11*B*), usually with a prominent nucleolus. The variable morphology of the nuclei of presumptive myoblasts with scattered or speckled electron dense chromatin perhaps denote transitional stages between the presumptive myoblast with dense granular nucleus and the satellite cell with nucleus having dense peripheral heterochromatin.

After the separation of the presumptive myoblast from the parent myofibre, by the formation of new cytoplasmic membranes between them, the presumptive myoblast will then be a new satellite cell, ready to promote muscle regeneration.

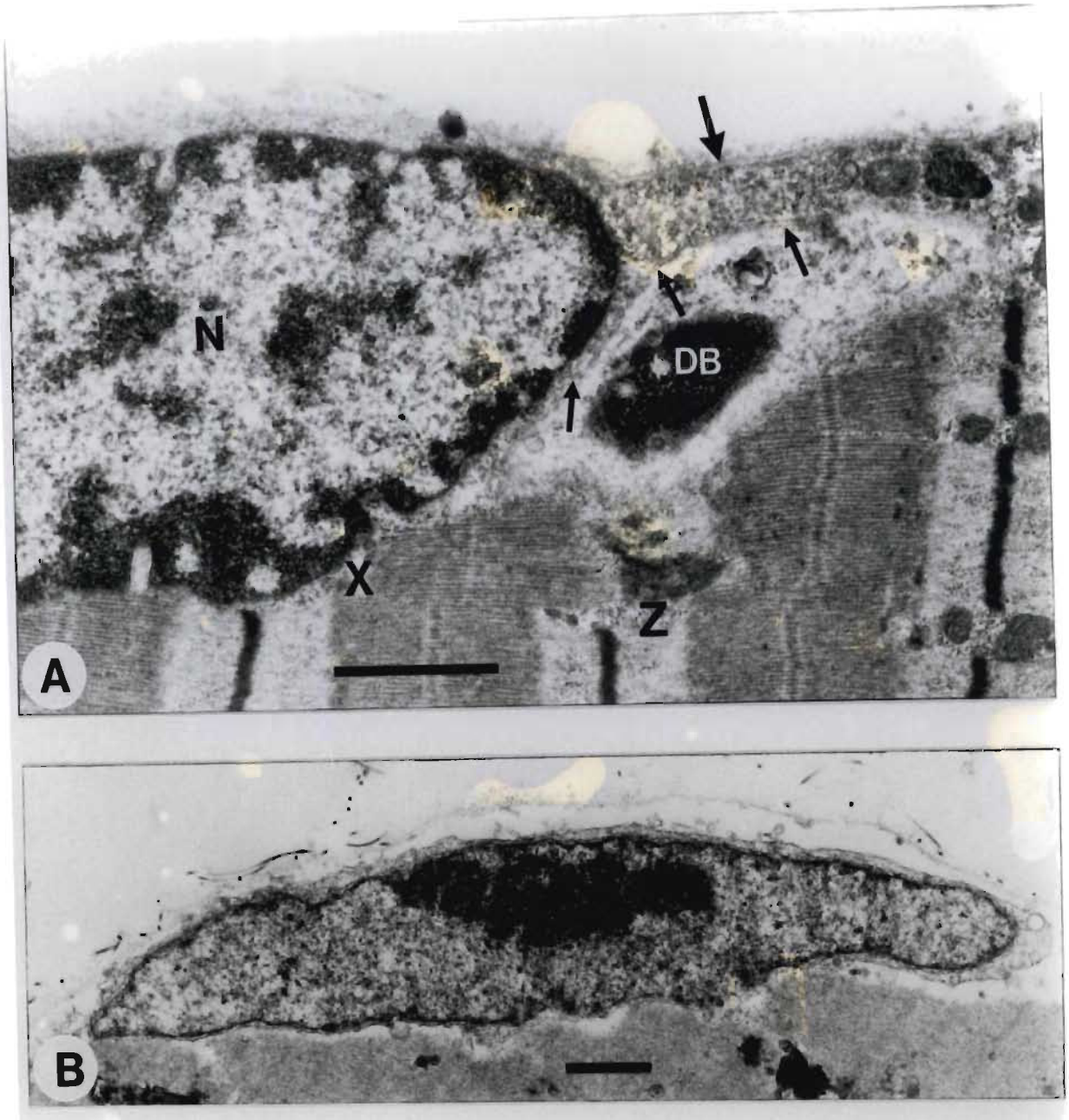


Figure 11. Electron micrographs of presumptive myoblasts in human explants up to 6 days incubation. *A:* Young cell developing from the myonucleus (N) below the basement membrane (thick arrow), with new electron dense cytoplasm development at one end of the nucleus. Distinct double membranes (thin arrows) reveal separation of the young presumptive myoblast cytoplasm from the sarcoplasm of the parent myofibre. There is no continuity of the membrane in the region marked X, showing attachment of the developing cell to the parent myofibre. Again a dense body (DB), opposed to the Z line which is disorganised, is found close to the transforming myonucleus. *B:* Full nucleus of a presumptive myoblast with little cytoplasm mainly at one pole of the nucleus. Bar = 1 μm.

During the incubation period, the myofibres, as explained before, underwent degenerative changes which included the disruption of the plasmalemma that remained as remnants along the basement membrane of the myofibre. Remnants of this plasmalemma remaining intact in segments of the myofibre, provide strong evidence for the derivation of a presumptive myoblast from a myonucleus, because the plasmalemma remnants positioned between the basement membrane and the presumptive myoblast in a number of instances, both in the hamster and the human explants suggested this (Figs. 12*B*, 12*C*, 13*A* and 13*C*).

That presumptive myoblasts are derived from myonuclei and from within the myofibre is further consolidated by the location of sarcoplasmic elements between the basement membrane and the cytoplasmic membrane of many of the presumptive myoblasts (Figs. 12*A* and 12*B*).

The supposition by many researchers that satellite cells fused with the subjacent myofibre was true for the process of myonucleation of muscle fibres in the normal course of myogenesis in the embryo, in the foetus and in the young. The above concept applied here in the case of regeneration, observed in muscle that has been experimentally injured and with the myofibres degenerating, would imply that these myoblasts from satellite cell origin, were trying to resuscitate the degenerate fibre. This was unlikely with the overwhelming evidence presented in regeneration studies which clearly indicated that restoration of damaged muscle was by the way of developing new myotubes (Vracko and Benditt, 1972; Carlson, 1973).

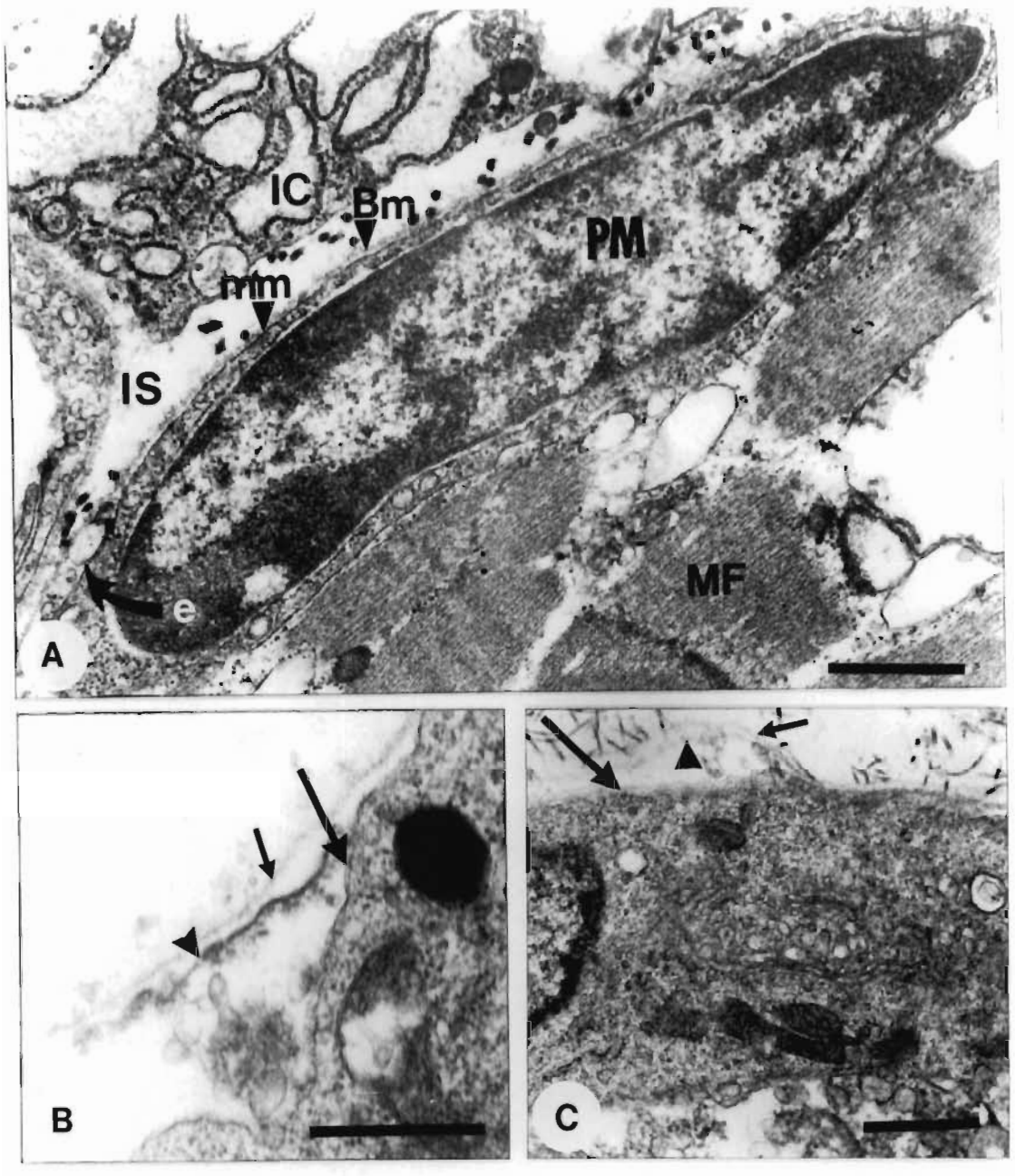


Figure 12. Electron micrographs of sublaminar cells in hamster explants up to 5 days incubation. *A:* Presumptive myoblast (PM) lying distinctly below the basement membrane, (Bm). Cytoplasmic elements (e) of the myofibre (MF), lying between the Bm and myoblast cytoplasmic membrane (mm), lends evidence that the myoblast originates within the myofibre syncytium. These presumptive myoblasts have scanty cytoplasm and organelle development. *B,C:* Showing remnants of plasmalemma (arrowhead) positioned in between the basement membrane (short arrow) and the cytoplasmic membrane of the presumptive myoblasts, lend further evidence that these cells originate from a myonucleus within the myofibre. IC, interstitial cell; IS, interstitial space. Bar = 0,5 μm in *A* & *B*, 1 μm in *C*.

The satellite cells, in the present study, were not observed in pairs. If satellite cells divided mitotically to give rise to new myoblasts then it would not be unreasonable to expect them to be found in pairs in at least some of the large number of myofibres inspected. On the other hand, myonuclei and sublaminar cells were often observed proximal to each other in the current study and by others (Schmallbruch and Hellhammer, 1977; Sandset and Korneliussen, 1978). Their proximity implies a possible derivation of sublaminar cells from the myonuclei. Furthermore, mitotic division of cells found in the sublaminar regions of the myofibres was not observed in the present study.

According to Konigsberg (1979) the solution to both the hypotheses of satellite cell versus the myonuclear derivation of precursor cells of regeneration were complicated by two difficulties. One was that muscle of animals experimentally injured were not observed soon after injury, but at progressively longer intervals after the time of injury. The other was that, the identification of fragments budding and the definitive identification of satellite cells, both required resolution afforded by the EM. Both these difficulties mentioned by Konigsberg were circumvented in the current study by tracing the early regenerative processes in cultured muscle explants with the EM.

The results of the current study strongly indicates that sublaminar mononucleated cells of mature muscle, which often are referred to as satellite cells were in fact myoblasts which were previously presumptive myoblasts derived from the myonuclei. Possible mechanisms of cleavage or segregation for myoblast development from myonuclei were already described (Lee, 1965; Hess and Rosner, 1970; Reznik, 1976; Hay, 1979).

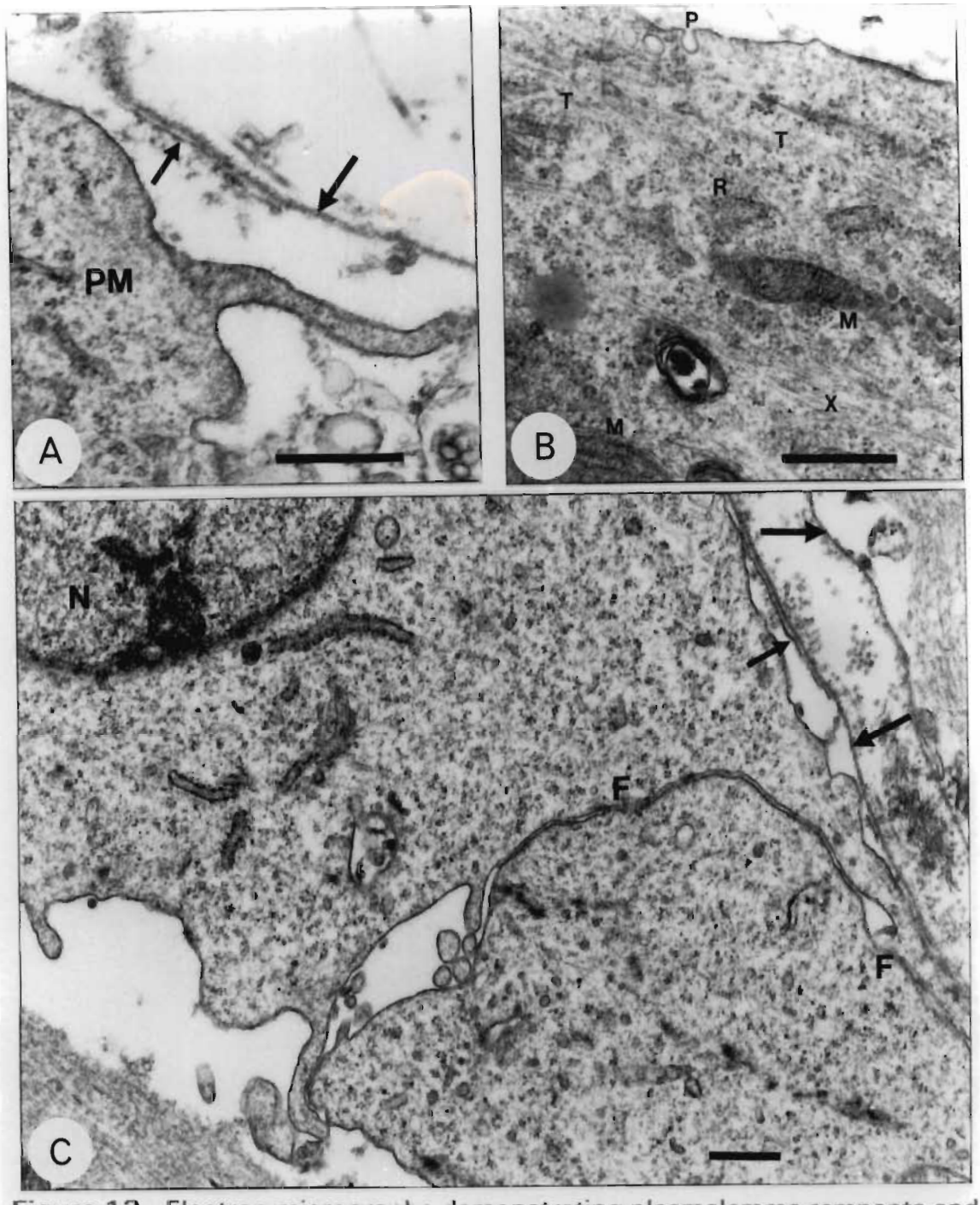


Figure 13. Electron micrographs demonstrating plasmalemma remnants and fusion of myoblasts in human explants from 3 to 10 days incubation. *A* & *C:* Parts of myoblasts with presence of remnants of the plasmalemma (short arrow) between the myoblasts and the basement membrane (long arrows) confirming the origin of these cells from within the sarcoplasm of the parent myofibre. *B:* Part of myoblast having pinocytic vesicles (P), microtubules (T), microfilaments (X), developing endoplasmic reticulum bordered by ribosomes (R) and developing mitochondria (M). *C:* Parts of 2 myoblasts coming together and fusing at points F where the membranes meet. N, nucleus. Bar = 0,5 μm.

But, the ultrastructural characteristics of the scanty cytoplasm of the presumptive myoblast, with little or no organelle development, as observed in this study, suggests that the cytoplasm may in fact be secreted by the active myonucleus in the initial stages of development of the myoblast. Light micrographs revealed mononucleated cells in regenerating myofibres to have intensely basophilic cytoplasm which was distinctly different from the subjacent cytoplasm of the myofibre. If myoblasts were formed by the cleavage mechanism (Hay, 1959; Hess and Rosner, 1970; Reznik, 1976) where the myonucleus with some cytoplasm separates from the myofibre by membrane formation between them, then the cytoplasm of both the new myoblast and the parent myofibre should have similar staining and structural features. Reznik (1969) also observing this difference, stated that "*Many of the myonuclei and their surrounding cytoplasm, however, have a different ultrastructural aspect from that observed in the undamaged region of the same muscle.*" Lash et al. (1957) also commented on the perinuclear cytoplasm of the young cells being basophilic.

5.5.2.4 Support for myonuclear derivation of myoblasts from other studies

There are other researchers (Hay, 1959/1979; Lee, 1965; Reznik, 1969/1976; Teravainen, 1970; Hess and Rosner, 1970; Walker, 1972) who believed that myonuclei had the potential to be activated in times of muscle stress. At these times of stress, myonuclei were said to surround themselves with sarcoplasm, and finally segregate as a new cell from the parent myofibre by a process called dedifferentiation (Hay, 1959/1979).

In vivo, regeneration studies (Allbrook, 1962; Lee, 1965; Reznik, 1969; Hess and Rosner, 1970) on rabbits, mice and guinea pigs, strongly support the findings of the present study. Allbrook (1962) described "*a type of cell commonly found in the position of a subsarcolemmal nucleus in a muscle fibre*". He went on to state that this cell had dense granular nucleus and it was "*otherwise similar to a subsarcolemmal muscle nucleus*". It was unfortunate that EM study was reported on, only after 90 hours of having caused injury to the muscle. It was likely that these subsarcolemmal cells would have been observed sooner had Allbrook resorted to electron microscopy earlier. However, in his review of 1981, he suggested satellite cells were the possible reserve myoblasts responsible for regeneration.

Lee (1965), investigating regeneration in gastrocnemius muscle of rat and rabbits, denervated for longer than three months, also supported the theory of the myonuclear derivation of muscle precursor cells. Lee, from his EM investigation, suggested that the cells in the periphery of myofibres were formed by invagination of the plasma membrane at the two poles of the myonucleus, with the membranes meeting and fusing, cordoning off the new cell thus formed.

Reznik (1969) in his study of muscle regeneration in adult rabbits and mice, also observed similar cells after 68 hours of injury. According to Reznik (1969), the myoblasts in the partially damaged part of myofibres appeared "*to be pinched off the muscle sarcoplasm as mononucleated cells that pass through a satellite cell stage*". In that study, it was stated that the majority of myonuclei in the degenerative phase of the fibres became pyknotic and disappeared. These pyknotic nuclei might be the dense granular nuclei referred to in this study, and perhaps their disappearance could be explained

by the fact that they transformed into cells. Reznik (1969) suggested that "one of the visible aspects of the production of myoblasts is the formation of plasma membranes that isolate a previously normal myonucleus and its surrounding envelope of cytoplasm. This process of membrane formation seems to occur by the progressive coalescence of vesicles and clefts, probably in connection with the extracellular space and presumably initiated in contact with the transverse tubular system". He went on to state that after these mononucleated cells were isolated from the myofibre, they proliferated by mitotic division. In his paper (Reznik 1969), electron micrographs of presumptive myoblasts with scanty cytoplasm still partially attached to the parent sarcoplasm were presented. Cells with a thin rim of cytoplasm enclosing an elongated nucleus were also observed in between necrotic myofibrils without other interstitial cells being present in the same area. Reznik further stated that satellite cells were not observed in the mouse and rabbit muscle before injury, "*despite an extensive search.*" This study by Reznik did not concentrate on the very early regenerative changes that occurred in the injured muscle otherwise it is certain that the very early transformational changes of the myonuclei would also have been identified.

Reger and Craig (1968) observed apparent fusion between myoblasts and underlying myofibres in hypertrophic human deltoid muscle and they suggested the possibility that satellite cells were fusing with the myofibres and thus causing enlargement. The alternate interpretation could also be that these cells were young presumptive myoblasts formed from myonuclear transformation with subsequent segregation of its new cytoplasm from the sarcoplasm. However, they did suggest that their speculation deserved continued study.

Teravainen (1970), to avoid degeneration and at the same time stimulate regeneration, caused slight compression injury to the musculi bulbi rectus superior of adult rats. The results of this study further supported the myonuclear derivation of cells during regeneration. Electron micrographs, revealed areas of protoplasmic contact between the so called satellite cells and the myofibre sarcoplasm which led Teravainen (1970) to conclude that the satellite cells originated from myonuclei by pinching off from the sarcoplasm. The satellite cells, with a pale nucleus having a prominent nucleolus and with few organelles in the pale cytoplasm were similar to many of the cells observed in the current study. Teravainen also made the point that satellite cells were never observed in close proximity to each other, but instead, "*the nucleolus of the satellite cell was usually observed close to the nucleus of the myofibre, suggesting that new satellite cells could be derived from the muscle cell nuclei.*" Furthermore, Teravainen reported that satellite cells increased in number during the first 10 to 12 hours after slight injury to the muscle. In culture conditions, the doubling time for cells was shown to be 22 hours. This fact then implies that the increase in the number of satellite cells so early after injury can not be attributed to satellite cell proliferation by mitotic division, unless the situation is different, ***in vivo,*** from that seen in cultures for doubling time of myoblasts.

Hess and Rosner (1970), working on denervated gastrocnemius muscle of adult guinea pigs, were of the opinion that satellite cells increased in number by the dedifferentiation or budding mechanism described by Hay (1959/1979) for newt regeneration of muscle. They identified many cells that were "*fused, joined, attached or part of the peripheral sarcoplasm of the muscle fibre. Several of the satellite cells can be seen for one short*

stretch to be free and for another short stretch to be part of the muscle fibre, as if they were caught in the act of being pinched off from the muscle fibre". They proposed that clefts appeared between the myonucleus and the rest of the muscle fibre, or else invaginations from the plasmalemma around the myonucleus finally meeting and forming a separation channel, was the means by which a new cell was formed from the myonucleus. Again the morphological descriptions of the partially formed cells was the same for the cells presented in this study on hamster and human muscle. Hess and Rosner (1970) also did not see any mitotic division of cells in the myofibres.

The dedifferentiation of myonuclei, segregating some cytoplasm around it and budding of new cells was thought to be similar to the mechanism of regeneration demonstrated in some amphibians and reptiles after amputation of their limbs (Thornton, 1938; Carlson, 1979; Hay, 1959/1979). According to Hay (1979), during the formation of the blastema, the contribution of mononucleated cells made by muscle, was by means of myonuclei dissociating or segregating with cytoplasm from the muscle. This mechanism where myonuclei segregated from the muscle fibre with sarcoplasm was termed dedifferentiation (Hay, 1959/1979). Furthermore, according to Hay, the adult newt had no pre-existing satellite cells in the muscle. Hay concluded that the muscle formation in the embryo by myoblast fusion was reversible in the adult newt when need for myoblast proliferation was imposed on a muscle. Also, that there was enough reason to research for this myonuclear derivation of cells in avian and mammalian muscle. Hay (1979) felt that an important message from the newt ought not to be ignored by concluding that satellite cell was a reserve cell without which muscle regeneration was not possible.

Walker (1972), using young rats subjected to radioactive isotopes in a double injury experiment where labelling was effected after injury, obtained results which led him to conclude that myonuclei were responsible for the development of myotubes during muscle regeneration. This was contrary to most of the other radioactive labelling studies which promoted the hypothesis that satellite cells gave rise to new myoblasts during muscle regeneration. Walker used the same labelling technique of injecting [^{3}H]thymidine into rats. In Walker's research, one group of rats was subjected to a single injury. In this group the muscle was injured 12 days after multiple injections of radioactive thymidine given over 3 days. The second group was subjected to a double injury, where [^{3}H]thymidine was injected into the rats 2 days after injury of muscle which was subjected to re-injury 12 days later. In both the experiments the rats were sacrificed 4 days after the last injury to the muscle. The absence of labels in the myotube nuclei in the single injury group implied that satellite cells were not involved in the formation of the myotubes. The incidence of labelling of the myotube nuclei and centrally located nuclei of maturing fibres in the second group led to the conclusion that myonuclei contributed the precursor cells which fused and formed the labelled myotubes after the second injury.

Recently, Ono et al. (1994), conducted a study to clarify proliferating cells, static cells and proliferative activity of myogenic cells in neuromuscular diseases. Muscle biopsies, from a number of dystrophic conditions, were subjected to monoclonal anti-K-67 (MIB-1) antibody as a specific marker. Mononucleated cells including myoblasts and macrophages showed positive reaction to MIB-1. Apart from this, Ono et al. (1994) observed that "*a small number of sarcolemmal nuclei, both of non-necrotic myofibres in myogenic diseases and non-atrophic (hypertrophic) myofibres in neurogenic diseases,*

were also positive for MIB-1". According to them, "*This finding suggests that sarcolemmal nuclei (possibly satellite cells) of myofibres without necrosis could go into the cell cycle under some unknown signals*". In a personal communication with Ono at the Neuromuscular Diseases Congress (1994), it was suggested that the MIB-1 positive sarcolemmal nuclei could have been the transforming nuclei referred to in the current study, and that they assumed that the labelled nuclei belonged to satellite cells because of the current belief in research circles that only satellite cells were able to synthesize new DNA and take up the markers.

5.5.3 The satellite theory on the derivation of myoblasts

It was suggested in numerous studies on regeneration that satellite cells, first described by Mauro (1961), were the muscle precursor cells responsible for promoting muscle regeneration (Church, 1969; Mastaglia and Kakulas, 1969; Moss and Leblond, 1971; Schmalbruch, 1976; Bischoff, 1979; Hsu et al., 1979; Konigsberg, 1979; Nichols and Shafiq, 1979; Ontell, 1979; Snow, 1979; Allbrook, 1981; Schultz et al., 1985; Carpenter, 1990). The satellite cells were thought to be formed by the basement membrane or basal laminar enveloping free myoblasts that were associated with the myofibre during the early development of muscle (Kelly and Zacks, 1969; Church, 1969; Ontell, 1974). However, it has not been proven conclusively that the satellite cells are the precursor cells of muscle regeneration (Sloper and Partridge, 1980). The acceptance by the many research stations that satellite cells were the precursor cells dampened research into regeneration in recent times.

Satellite cells, supposedly being remnants of embryonic myogenesis, lying between the basement membrane and the plasmalemma of the myofibre, were said to behave as stem cells when the adequate stimulus to promote muscle regeneration was made available (Moore, 1979). Characteristically, these satellite cells ought to have dense peripheral heterochromatin with scanty cytoplasm. However, most of the regeneration studies seemed to consider most sublaminar mononucleated cells, irrespective of their morphological characteristics, as satellite cells leading to much controversy in literature (Reznik, 1976).

Some of the conclusions drawn from the results obtained from the many regeneration studies supporting the satellite hypothesis are debatable and far from being conclusive! A few of the pertinent studies advocating the hypothesis that the satellite cells are the precursor cells of muscle regeneration are discussed below.

Bischoff, in his research (1979), performing a myogenic cell suspension study with muscle treated with enzymes that digest the basal lamina and with those that do not, did not in any way identify precursor cells of regeneration. Cells isolated by trypsin digestion of muscle when cultured formed myotubes and cross striated myofibres. Those enzymes that did not digest the basal lamina released non-myogenic cells which in cultures produced fibroblasts, fat cells and macrophages. The deduction from this experiment was that the myogenic cells released from the sublaminar position were myogenic stem cells or satellite cells. There was no proof of the origin of the myogenic cells mentioned by Bischoff (1979), except that they were cells obtained from the sublaminar regions of the myofibre. To have referred to those myogenic cells as stem cells or satellite cells would

be erroneous because an alternate deduction could also be that precursor cells formed by transformation of myonuclei may have been the myogenic cells cultured.

In another experiment with single fibres cultured in a fibrin clot, Bischoff (1979) reported clot retraction of the fibres with clear zones between the clots, containing myogenic cells which he proposed as derivatives of satellite cells that had proliferated. Furthermore, electron micrographs presented by Bischoff, taken at zero time in culture, revealed gross degenerative features and myonuclei were said to have disappeared supposedly due to degeneration. The interpretation or identification between interstitial and satellite cells as well as between the basal membrane and plasmalemma of the myofibre presented in that work was debatable. It would have been interesting had he examined the clot itself with the EM because it was possible that the myonuclei were lodged in them during retraction. Again, the pre-existing cells found within the clear zones between the clots were assumed to be satellite cells.

Konigsberg (1979) continually monitored cultured single muscle fibres of quails with a phase contrast photomicroscope. The culture technique of single fibres, in present day terms, was obviously inefficient as can be interpreted from some of the results obtained in that study. He reported that all the myonuclei from the cultured myofibres had disappeared. Furthermore, from 505 fibres cultured, only 110 gave rise to some cells and only two colonies of mononucleated cells were formed. At the outset in the cultures, many of the surviving single cells were reported having a tangential orientation rather than parallel to the fibre axis. The results of the above study did not prove anything conclusively as Konigsberg (1979) suggested

satellite cells were the precursor cells, and at the same time, he did not exclude the possibility of budding of new cells from myonuclear origin. Again, Konigsberg also did not trace the origin of the cells. The assumption was made on the basis that spindle shaped cells that emanated from the ends of cultured myofibres must be satellites without any concrete evidence. The main body of support for satellite cells being myogenic precursors came from [^{3}H]thymidine studies (Moss and Leblond, 1970/1971; Hsu et al., 1979; Snow, 1979).

Only satellite cells were considered responsible for the contribution of new nuclei to growing muscles of young rats injected with radioactive thymidine (Moss and Leblond, 1970/1971). In the latter study, from the results shown in the table below,

Time elapsing between thymidine-^{3}H injection and sacrifice - *h*	No of nuclei labelled	
	Satellite cell nuclei	True muscle nuclei
1	20	0
6	11	0
10	24	0
24	8	2
48	12	11
72	4	24

Over 300 nuclei examined/animal - Moss and Leblond (1970)

no myonuclei were labelled 1, 6 and 10 h after injection of the [^{3}H]thymidine, but 20, 11 and 24 satellite cells respectively, were labelled. It is from these results that Moss and Leblond (1970) have drawn their conclusion. They did not explain why at 6 h after injection the satellite count dropped to 11 and then again rose to 24 after 10 h. If satellite cells,

in time, were taking up the labels then there ought to be a consistent rise. On the other hand, if one takes the dilution factor of labels into account then there would be a consistent decline in the number of satellite cells labelled as the time increased.

At 24, 48 and 72 h after injection, there was a consistent rise in the number of myonuclei that were labelled. According to Moss and Leblond (1970), the explanation for the rise in the number of labelled myonuclei was that these myonuclei probably belonged to satellite cells that had already fused with the fibre. If rapid fusion process between the satellite cells and the myofibre was suggested, then how could they account for no myonuclei taking up the labels before 10 h after injection - certainly some cells must have been undergoing fusion with the myofibre at this time. It seemed remote that all these labelled satellite cells would have lost all their cytoplasm in such short time to assume the identity of true myonuclei. An alternate explanation may be that these labelled myonuclei were the activated dense granular myonuclei which underwent transformation towards becoming a new presumptive myoblast which perhaps assist with regeneration of the muscle subjected to the toxic effects of the isotope itself. The myonuclei must have taken the labels because their must be new DNA formed during their activation or transformation.

Hsu et al. (1979) observing the presence of labels in myotube nuclei derived from cultured explants of neonatal rat muscle labelled with [^{3}H]thymidine concluded that satellite cells were the myogenic elements in, ***in vitro,*** myogenesis and not myonuclei. Unfortunately, the explants were not examined to identify the derivation of new cells. Instead labelling was inspected in the myotubes that formed from the young cells which sprouted

from the explants. The labelling protocol rested on the labelled neonatal satellite cells fusing with adjacent myofibres to produce increased number of myonuclei. Only satellite cells (no figures given) were said to be labelled after 10 h injection. By 24 h, 15% of the myonuclei were labelled in the neonatal rat muscle. Actual figures of satellite cells and myonuclei labelled were not given to make any useful interpretation or further comment on this aspect of their study. Moreover, the animals being neonatal rats, were still very young and in the growth phase where almost all the satellite cells in this active state ought to be labelled.

Hsu et al. (1979) went on to state that the labelled nuclei of myotubes from the explant cultures were obtained from labelled satellite cells. According to them, 25 to 30% of the myotubes contained labelled myonuclei. A question that immediately comes to mind is: where did the nuclei of the other 70 to 75% of myotubes come from? If the explants were taken from neonatal rat muscle 8 h after injection of the labels, most, if not all, of the satellite cells ought to be labelled. Therefore, if the argument is that pre-existing satellite cells contributed nuclei to the myotubes, then most of the myotubes ought to have revealed labelled nuclei.

Snow (1978/1979) attempted differential labelling of myonuclei and satellite cell nuclei with three experimental groups of young rats to gain clarity on the origin of myoblasts during regeneration. Both the results and the conclusions drawn from these experiments are debatable. In the first group, [^{3}H]thymidine was initially injected into pregnant rats with subsequent injections administered to the new born to effect labelling of myonuclei that would have occurred during the fusion and maturation of the myofibres in

the young. After 5-6 weeks maturation, muscle was injured by mincing and autografted. After 8-24 h, thick and thin section inspection of the regenerates revealed labels in 20% of the pyknotic nuclei but not in the viable mononucleated cells which made up 6% of the total sublaminar nuclei. The same percentage of myonuclei were labelled in the uninjured controls but the satellite cell nuclei were over 11%.

If one considers the fact that satellite cells were supposed to be reserve myoblasts lying under the basement membrane, then at least a few of them ought to have been labelled, even if one had to take the dilution factor into account. The death of all the myonuclei in the autografts also need an explanation. Perhaps the pyknotic myonuclei described by Snow (1978/1979) are the dense heterochromatic myonuclei which show signs of activation and transformation in the current study. Furthermore, the reason for the decreased number of mononucleated cells in the regenerates was not clear. If anything, the satellite cell number ought to have increased in the regenerates. Unfortunately the animals were subjected to the isotopes for several days preceding the regeneration of muscle resulting from injury. Perhaps, labelling at the time of regeneration, after injury, would have produced a different picture.

In the second group, labelling of satellite cells was effected in uninjured muscle of 15-17 day old rats which had a relatively high satellite cell count of about 15%. One hour after a high pulse dose, muscle was minced and autografted as before. Results in regenerates, 8-24 h after injury, showed 23% of sublaminar mononucleated cells labelled, tallying with the labelled satellite cell counts before injury. Again, a question arises as to why only 23% of satellite cells took on the labels before injury if these were young

animals with active satellite cells, and after injury one would expect the so called reserve satellite cells to be activated and would therefore expect a higher number of satellite cells labelled and not the same number as the uninjured muscle. It is well documented that the number of sublaminar cells increases in regenerates. In this group, a number of pyknotic nuclei were also labelled and the reason for this was not adequately answered.

For want of better interpretation, Snow presumed that these labelled pyknotic nuclei belonged to satellite cells that degenerated after undergoing fusion with the myofibre. Furthermore, it was difficult to accept that satellite cells fused with degenerate myofibres as suggested in the above studies. This interpretation meant that satellite cells make a suicidal attempt to resuscitate degenerate myofibres. However, an alternate interpretation of the results of Snow (1979) will point to transforming myonuclei taking up the labels indicating they were the precursors of myoblasts. The labelled nucleus shown by Snow (1979) was most likely an activated dense granular myonucleus on its way to become a myoblast, and the many large mitochondria in close association with it denoted its energy requirement for such a transformation.

The third group of rats was used to test the survival and differentiation of the satellite cells. Labelled muscle mince was implanted into nonradioactive littermates and examined 4 to 6 days later. Approximately 7% of the nuclei of myotubes were labelled even though control muscle had a count of 31% labelled satellite cells. The dilution of labels during mitotic division as well as possible death of some of the satellite cells were forwarded as the reason for the discrepancy in the labelling indices. Snow (1979) concluded that satellite cells were capable of surviving injury to promote regeneration, but

that "*the role of satellites in adult skeletal muscle regeneration remains uncertain*".

As explained earlier, Konigsberg (1979) mentioned the fact that interpretation of the results of these regeneration studies were made difficult by animals being experimentally injured and then being observed at progressively longer intervals after the time of injury. Another difficulty would appear to be that many of these studies seemed to have been performed with a foregone conclusion that only satellite cells synthesize new DNA and that it was not possible for myonuclei to become activated and also synthesize new DNA. If the latter was accepted as a possibility, then much of the results obtained by autoradiographic studies could be explained without incurring confusion. At this point, it is worth remembering that in another labelling study (Walker, 1972), it was concluded that myonuclei were responsible for the development of new myotubes during regeneration.

In another study (Anderson et al., 1987) using radioisotopes on mdx mice of different ages it was shown that there was an increase in the number of sublaminal nuclei in the 32 wk old mdx muscle than in the normal 4 wk age group. The results of that study did not indicate whether the labels were taken up by satellite cells or by myonuclei. The reason for this was that the researchers in the above study presumed that only satellite cells take up labels. Their results also showed that in 4 wk old mdx mouse muscle the labelled nuclei number increased at 48 h after injection of the labels by nearly 6 times the number counted for 2 h after injection. In the 32 wk old mdx mouse muscle, the number of labelled nuclei counted after 48 h was more than twice the number counted at 2 h after injection of the labels. Anderson et al. (1987) were aware that the counts indicated values more

than that which could be explained by the doubling time for cells by mitotic division. However, they were unable to supply a suitable reason for this discrepancy in counts nor could they explain why the older mdx mouse muscle had more labels than the younger normal control animals. From the findings of the current morphological study, on the regenerative events in muscle, the result obtained by Anderson et al. (1987) point in the direction that new cells from myonuclear derivation and activated myonuclei were responsible for the discrepancy in the results obtained by them. It was unfortunate that Anderson et al. were of the notion that only satellite cells of embryonic origin prevailed in the muscle and that was the only source of new DNA in muscle that can take up the labels.

A question that needs to be asked is - which of the labelling results and conclusions in the different studies mentioned are correct?

Satellite cells were shown to increase dramatically at local sites of injury (Schultz et al., 1985). Seeing that the number of satellite cells at these sites can not be explained mathematically by satellite cells proliferating by mitotic division alone, i.e., when taking into account the doubling time of the cells, migration of satellite cells was proposed as the reason for the large number of cells at the sites of injury. Reznik (1976), having compared research results in a review on the origin of the myogenic cell, was of the opinion that myoblasts could be derived from pre-existing satellite cells. On the other hand, Reznik (1976) felt that it was difficult to accept that, ***in vivo***, so many myoblasts resulted from so few satellite cells in such a short time after local injury and therefore, myoblasts were probably produced from other sources in the myofibre.

5.5.4 Summary of evidence for myonuclear derivation of myoblasts with some comments

Sufficient evidence in the present study, to strongly support and accept the theory that precursor cells or presumptive myoblasts, which lead on to myoblasts, arises from myonuclei during muscle regeneration, is as follows:

a. The transformation of euchromatic myonuclei to electron dense granular myonuclei in regenerating myofibres. Many of the other research studies found nuclei to have become pyknotic or disappeared altogether. The presence of myonuclei for much of the incubation period in the current study shows the advantage of this method of studying regeneration compared to the others.

b. A decline in the number of myonuclei with a definite increase in the number of presumptive myoblasts as regeneration proceeds would intimate that myonuclei were being transformed into cells. Almost all myofibre nuclei observed between 3 to 6 days of incubation belonged to either mononucleate or multinucleate cells.

c. The presence of dense granular nucleus in the presumptive myoblasts resembling the dense granular myonucleus.

d. The observation of cytoplasm of different electron density around one pole of a myonucleus from that of the sarcoplasm without any visible membrane separation between the two plasmic regions denote the early formative stage of a presumptive myoblast.

e. The observation of scanty cytoplasm with little or no organelle development, occurring adjacent to one pole of the myonuclei, beginning to develop membranes which reveal separation lines between the presumptive myoblast and the sarcoplasm of the parent myofibre. These early stages of myonuclear transformations were not observed or mentioned in any of the articles reviewed.

f. The location of the presumptive myoblast with scanty cytoplasm within the myofibre without visible complete separation between the two by a plasmalemma of the parent myofibre. Some presumptive myoblasts had a nucleus that was identical to a satellite cell nucleus denoting that the presumptive myoblast was a stage preceding the development of the satellite cell.

g. The observation of the plasmalemma of the parent myofibre between the outer basement membrane and myoblast cytoplasmic membrane clearly points to the location and derivation of the presumptive myoblast being within the myofibre. This also was not recorded in any of the articles reviewed.

h. The presence of cytoplasmic elements of the parent myofibre occurring between the basement membrane and the myoblast cytoplasmic membrane also points to the derivation of the presumptive myoblasts from within the myofibre.

i. The line up of vesicles, which probably assist in membrane formation between the myoblast and the myofibre elements, seen in regions showing partial separation between the two.

j. The presumptive myoblasts occurred singly and were never observed in pairs in the early stages to indicate that they arose by mitotic division of the satellite cell. No previous studies have seen sublaminar cells occurring in pairs to validate that these cells arose by mitotic division.

k. Mitotic division was not observed in the sublaminar cells in the current study and, even by several other workers.

l. In most of the control muscle, classical satellite cells were rare. Another worker in an intense EM study searching for satellite cells in normal muscle did not find any (Reznik, 1969).

5.5.5 Sarcoplasmic morphology of explants

Whilst the incubated specimens showed signs of degeneration of the sarcoplasmic contents, the myofibrillar material was present in the myofibres through out the experimental period. As incubation time progressed, Z lines began to disappear (Shear, 1979). Z line distortions with Z line streaming were not uncommon in the myofibrils (Figs. 11*A* and 14*D*). The Z line streaming shown in Figure 14*D* closely resembles tubular aggregates sometimes seen in myotubular myopathy (Sewry, 1985). Streaming (Kimura et al., 1990) and twisting (Lichtig et al., 1993) of the Z line were common in pathological tissue. Z line streaming and disorganisation were also observed in the muscle of patients complaining of exertional myalgia (Isaacs and Badenhorst, 1992)

Cytoplasmic bodies (Figs. 8*D*, 11*A*) were commonly observed in both hamster and human muscle explants after incubation. They were also observed in human control specimens. These electron dense cytoplasmic bodies appeared very much like a nucleus sectioned tangentially at the electron dense region, demonstrating pore like structures. They did not seem to have any limiting membranes and they were usually found opposed to and involving the Z line material. The cytoplasmic bodies were invariably associated with smooth microvesicles which perhaps were the finer parts of the sarcoplasmic reticulum. Sewry (1985) refers to these microvesicle like-structures as nuclear pores in a somewhat similar electron micrograph of a section through the nuclear envelope. But in that same micrograph, similar structures referred to as nuclear pores are also seen out of the nuclear envelope in the adjacent sarcoplasm. It would therefore be incorrect for Sewry to refer to these structures as nuclear pores. Incidentally, the part of a nuclear structure shown by Sewry is also apparently associated with the Z line.

The mitochondrial morphology of the myofibres in the explants showed great variation in both the hamster and human muscle, before and after incubation. As incubation progressed, the mitochondrial number increased. Increase in mitochondrial number was also observed in muscle of albino rats subjected to subtle injury by exposure to incandescent and fluorescent light energy (Shear, 1979). Degenerate mitochondria, with crystal membrane disappearance leaving mitochondrial ghosts behind, were commonplace in muscle explants (Mendell et al., 1972). Many of the mitochondria were swollen (Figs. 14*A* and 15*A*) containing dense osmiophillic bodies and longitudinal paracrystalline inclusions in the intracrystal space as well as between the outer and inner membranes. Swollen mitochondria (Fig. 14*B*)

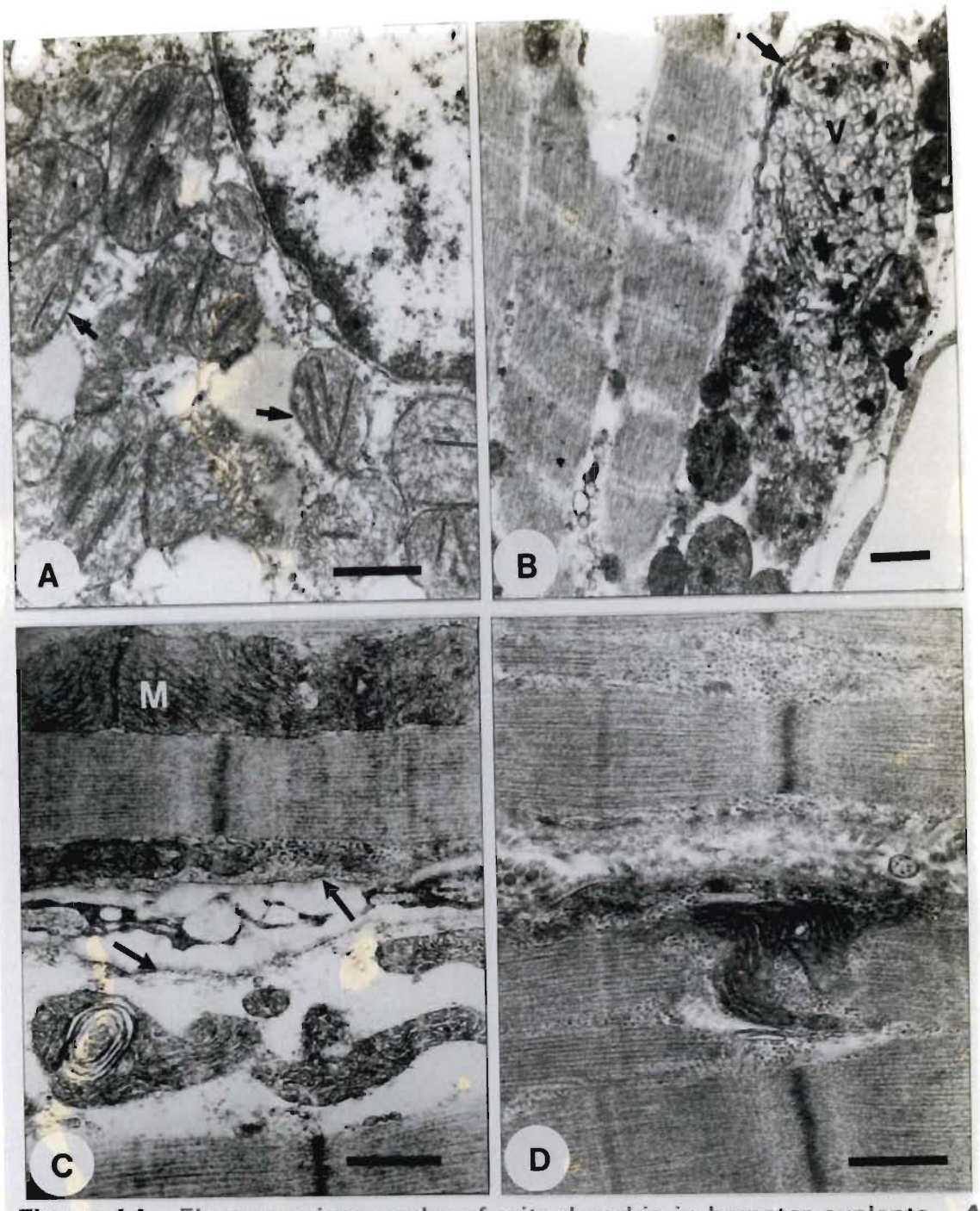


Figure 14. Electron micrographs of mitochondria in hamster explants. *A:* Mitochondrial changes with bars of paracrystalline substance in the intercrystal space and in between the outer and inner membranes (arrows) in control and incubated specimens. *B:* From 3 days incubation, some large mitochondria with vesicles (v), and organisation of membranes (arrow) with paracrystalline substance, on the periphery of the mitochondria. Dense osmiophilic bodies were a common finding in the mitochondria of the degenerating myofibres. *C:* Intermyofibrillar, longitudinal mitochondria (M) showing disruption, and a subsarcolemmal pleomorphic mitochondria with a myelin-like body. Arrow, basement membrane. *D:* Z line disorganisation or streaming in some intact myofibres in culture. Bar = 0,5 μm.

filled with vesicles with apparent reorganisation of the crystal membranes were seen after incubation. Cluster of vesicles were often seen in the neighbourhood of vesicular mitochondria denoting that they may have been derived from mitochondria. Mitochondria found in the intermyofibrillar space usually appeared compressed and longitudinal (Fig. 14*C*) when there was an obvious competition for space. Unusual shapes or pleomorphic and long mitochondria (Figs. 14*C* and 15*C*) were not uncommon in the muscle explants.

Concentric membrane formations within the mitochondria (Fig. 14*C*) similar in appearance to some myelin bodies described in pathological tissue (Nonaka et al., 1981; Matsubara and Tanabe, 1982; Isaacs et al., 1988) were sometimes seen in the incubated explants. It would appear that some of the pleomorphism in mitochondria was due to fusion of the mitochondria (Fig. 14*C*). Fusion of mitochondria was also demonstrated in the flight muscle of the blow fly, and incidently, the flight muscle of insects were considered suitable for muscle development studies as the sequence of development and senescence was completed in a matter of days (Gregory et al., 1968). As incubation time increased, there was a progressive proliferation of mitochondria in the sarcoplasm mainly in the subsarcolemmal regions, appearing in clusters (Fig. 15*B*). More will be said about these proliferating mitochondria later in the discussion.

Pleomorphic forms of mitochondria with crystal vesiculation, myelin figure formation, dense osmiophillic bodies and paracrystalline substances in bar formation were seen in a variety of disorders (Price et al., 1964; Papadimitriou and Mastaglia, 1982; Isaacs and Badenhorst, 1992).

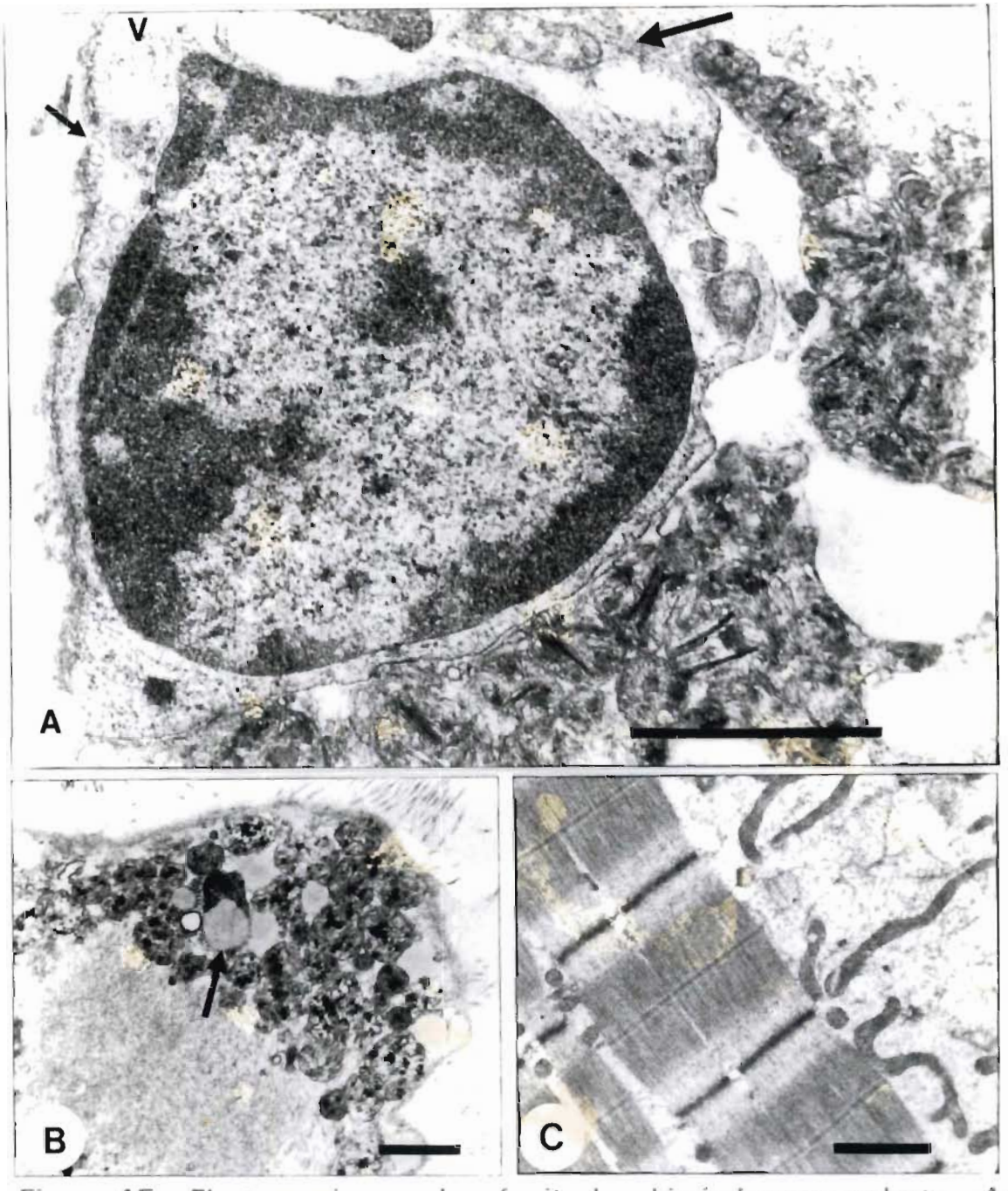


Figure 15. Electron micrographs of mitochondria in human explants. *A*: Fusing mitochondrial aggregations, with paracrystalline bars and dense osmiophilic bodies adjacent to a presumptive myoblast still attached to the sarcoplasm of a parent myofibre (long arrow) (3rd day). Many vesicles (V) are seen along parts of the cytoplasmic membrane of the young cell. *B*: Mitochondrial cluster with lipid bodies in a sublaminal region of a degenerate myofibre seen on the 4th day. A dense vacuolated cytoplasmic body (arrow), similar to those often observed near transforming myonuclei and young presumptive myoblasts, is present amongst the mitochondria. *C*: Elongated and pleomorphic mitochondria observed in some explants before incubation. Bar = 1 μm.

Mitochondria were sometimes decreased in numbers (Mastaglia and Kakulas, 1969) and swollen and degenerate in DMD (Atkin et al., 1991). Mitochondrial elongation in distal myopathy (Isaacs et al., 1988) and accumulation in degenerating diaphragm muscle fibres (Kimura et al., 1990) were also reported. Numerous, large and bizarre mitochondria were seen in oculopharyngeal muscular dystrophy (Pauzner et al., 1991) and in mitochondrial myopathy with clinical characteristics of progressive muscular dystrophy (Nagaura et al., 1990). Similar mitochondrial abnormalities prevailed in cardiac muscle of DMD patients (Wakai et al., 1988).

Although structural variation of mitochondria have been demonstrated in many instances, no specific biochemical defect has been associated with the different structures in the several biochemical studies (Morgan-Hughes, 1982 - cited in Dubowitz, 1985).

Typical Golgi complexes were not observed in the sarcoplasm in the current study, and if they were there, it was difficult to distinguish between them and the dilated sarcoplasmic reticulum often observed proximal to the myonuclear regions of the myofibres. Allbrook (1962) also stated that the regenerating myofibre did not reveal any obvious evidence of Golgi complex.

Granular endoplasmic reticulum were not observed in the sarcoplasm of the myofibres. Sarcoplasmic reticulum of various diameters were present in the subsarcolemmal regions as well as in the intermyofibrillar space. The sarcoplasmic reticulum seemed to become dilated as incubation time progressed. In the intermyofibrillar space, sarcoplasmic reticulum with the pale-appearing T-tubules in between them, were observed mainly in the control samples. During the early incubation period where the myofibrillar morphology was reasonably intact, the terminal cisternae together with the

T-tubule appeared dilated. The triad pattern became disrupted as incubation proceeded due to the myofibrillar degeneration and disorganisation. Dilated profiles of sarcotubular system were also observed in the ocular muscle of albino rats exposed to incandescent and fluorescent light (Shear, 1979), and in the muscle of young mdx mice (Anderson et al., 1987).

Most of the muscle explants used in this study were apparently normal, especially those taken from the hamsters. Yet, many of the structural changes in the sarcoplasm, namely, that of the mitochondria, the dense nuclear-like bodies, the dilated sarcoplasmic reticulum and the Z line distortions and streaming, observed in this study on regeneration, are changes often seen in pathological muscle. Therefore, their presence in pathological muscle perhaps signals regeneration.

5.5.6 EM morphology of sublaminar cells in cultured explant myofibres

The sublaminar cells observed in the incubated explants included the satellite cells, the cells that were still partially formed from myonuclei, the mononucleated cells or presumptive myoblasts that did not have the classical characteristics of satellite cells and the cells that were binucleate or multinucleate myotubes. The cells that were still in the formative stages have already been discussed. This section does not include the "new generation cells" which are discussed as a separate issue.

The satellite cell counts in the hamster thigh muscle ranged between 4 to 10% with an average of 7% (Table 3). This count compared favourably with the findings of other workers (Allbrook et al., 1971; Snow, 1979;

Wakayama and Schotland, 1979; Ishimoto et al., 1983) using different animals. In the human explants satellite counts ranged between 0 to 7% with an average of 2.6% (Table 4). Many of the cells counted, strictly speaking, did not conform to the characteristic requirements for classification as satellite cells. Regenerative features showing cells still attached to the sarcoplasm were observed in the hamster and the human muscle, but more so in the human muscle specimens. The possible reasons for regenerative features prevailing in the human muscle have been mentioned earlier. The time delay of between 2 to 4 h before the control explants were prepared from the hamster muscle perhaps was sufficient time to initiate regenerative changes in the muscle. If one had to disregard all the cells that did not have a nuclear morphology of the classical satellite cell described by Mauro (1961) and those that were still attached to the sarcoplasm, the counts in the human samples would have been much lower.

Unfortunately, satellite cell counts to compare for the different days of incubation were unreliable. This was on account of the fact that different explants from the same culture for any given day of incubation did not reveal the same level of differentiation, with some explants showing more regenerative features than others, clearly indicating that the regenerative process was asynchronous from explant to explant. This variation can possibly be accounted for by the different sizes of each explant and the varied levels of mechanical injury sustained during their preparation for culture. However, it was quite safe to estimate the sublaminal cells (satellite cells) to range between 10 to 60% for the hamster muscle between 1 and 3 days of incubation. The rate of increase in the number of sublaminal cells in the human muscle after incubation was much slower and showed greater variation in numbers for the different types of muscle used. Nevertheless,

sublaminar cells in the human explants also increased in number during incubation. As the number of sublaminar cells increased, the number of myonuclei in the muscle explants decreased.

Schmalbruch and Hellhammer (1977) showed that a relationship existed between satellite cell number versus myonuclei for a given volume of muscle such that if myonuclear number increased, the satellite cells would show a proportional increase. Because of the great difference between the satellite cells and myonuclear numbers, this relationship between satellite cell number versus myonuclei number suggested by Schmalbruch and Hellhammer, would imply that satellite cells arose from myonuclei.

Apparently, the population of satellite cell was closely tied to that of the myonuclei within the myofibre (Kelly, 1979) and were considered by many to behave as stem cells (Moore, 1979). However, satellite cells were not observed in pairs in the current study, but it was not uncommon to find satellite cells near myonuclei, and this was also observed in other studies (Ontell, 1974). Hansen-Smith et al. (1979) researching satellite cells in malnourished and clinically treated children also observed satellite cells and myonuclei proximal to each other. According to the findings in the above study, the myonucleus and the satellite nucleus had similar morphology whenever seen together i.e. both were either euchromatic or heterochromatic, and furthermore, satellite cells were also observed partially joined to the parent myofibre in the latter study. The number of satellite cells present in a given muscle depends on the physiological state of the muscle, with greater number being present in young and growing animals as compared with the old, in which case satellite cells are scarce (Campion, 1984). If satellite cells were considered embryonic remnants, then they

ought not increase or decrease in number after cessation of muscle growth in the adult. Nevertheless, satellite cells were seen in muscle of a 73 yr old man (0,6%) with human adult average being 4% (Schmalbruch and Hellhammer, 1976). In the current study, although the human muscle revealed early regenerative changes, the satellite cell count was much lower than that indicated by Schmalbruch and Hellhammer. This, perhaps, was due to the fact that different muscle samples were used in the current study. In the current study, a 65 year old man also had satellite cells. The differences in distribution of satellite cells in different muscles in the same animal, as shown for the soleus and the extensor digitorum longus muscles of the rat, were attributed to a distinction laid down in early myogenesis (Kelly 1978b).

The different stages of presumptive myoblasts with variable nuclear morphology having different euchromatic and heterochromatic pattern and distribution, observed in this study, were also seen in other works (Ontell 1977/1979; Schiaffino et al., 1979; Wakayama and Schotland, 1979; Dubowitz, 1985). As mentioned previously, the nuclear morphology of the sublaminar cells, invariably, resembled the morphology of the myonuclei that were earlier described as transforming or activated myonuclei. This perhaps was one of the indications of the derivation of the sublaminar cells.

The nuclei of some of the sublaminar cells displayed nuclear inclusions that were similar to small vesicles and fibrillar material, similar to that described by Anderson et al. (1987). Perhaps these were obtained by the nuclei from imbibition of cytoplasmic elements discussed earlier in this chapter. Deep invaginations of the nuclear membranes trapping cytoplasmic contents within the nucleus was also observed in other studies (Mastaglia and

Walton, 1971). Cultured muscle from oculopharyngeal dystrophy patients also revealed nuclear inclusions (Tome et al., 1989). Various other types of nuclear inclusions have been identified in different studies: oval and rod shaped, closely packed fibres in dermatomyositis (Banker, 1975); membranous structures in perinecrotic myofibres in frog sartorius muscle (Rumyantsev, 1979); tubular inclusions (Tome and Fardeau, 1980; Bouchard et al., 1989) and filamentous inclusions (Coquet et al., 1983; Anderson et al., 1987; Coquet et al., 1990) in oculopharyngeal muscle dystrophy. The imbibition of degenerate myofibrillar material by the invaginated nuclei shown in the current study perhaps explains the number of filamentous structures observed by others mentioned above.

The nuclei of many of the more mature monucleated cells exhibited morphology that apparently signified possible amitotic division. This will be discussed later.

The cytoplasmic content of the sublaminar cells observed in the current study was variable and many of the sublaminar cells cannot be classed as satellite cells described by Mauro (1961). Instead, these mononucleated cells fitted the description of presumptive myoblasts, although many workers refer to all cells seen in the sublaminar position as satellite cells (Schmalbruch, 1978; Franzini-Armstrong, 1979; Hansen-Smith et al., 1979; Mazanet et al., 1982). The presumptive myoblasts were fusiform having short or long cytoplasmic tails which extended for considerable distances below the sarcolemma originating from either end of the elongated nucleus. Cytoplasmic projections like pseudopodia commonly crept in between the degenerate myofibrillar material. These cells with cytoplasmic projections often appeared to resemble phagocytic cells.

The cytoplasmic organelle development and the content of the presumptive myoblast apparently varied with the amount of cytoplasm in the cell. As discussed earlier, the very young cells which apparently transformed from myonuclei had very little cytoplasm, usually at one pole of the nucleus, with very scanty organelle structures, and at times none that were recognizable. As the cytoplasmic content increased, so did the identifiable organelles. Well developed mitochondria, endoplasmic reticulum, Golgi complexes and ribosomes were commonly seen in the more developed presumptive myoblasts (Figs. 12*C* and 13*B*). Ribosomal chains were sometimes observed in the young cells which seemed to herald the development of the granular endoplasmic reticulum. Ribosomal clusters, similar to that observed in other studies (Schultz, 1976; Campion, 1978; Nichols and Shafiq, 1979) were also a common feature in the more developed presumptive myoblasts. According to Larson et al. (1969), there was a consistent and orderly relationship between polyribosomes and myosin development.

Some of the developed myoblasts had microfilament and microtubules (Fig. 13*B*) which apparently seemed to announce the development of the presumptive myoblast (which are commonly referred to as activated satellite cells) to a true myoblast which showed signs of developing myofibrils as the next stage in the development. Microfilaments and microtubules were observed in mononucleated sublaminar cells by others as well (Nichols and Shafiq, 1979; Campion, 1984). Cytoplasmic inclusions such as autophagic vacuoles, electron dense cytoplasmic bodies, phagosomes and lipid bodies were observed in some of the cells, especially those that displayed phagocytic morphology. In fact some researchers considered cells with this morphology to be phagocytes (Trupin et al., 1979). The cytoplasmic borders, opposed to the basal laminar in a number of cells displayed

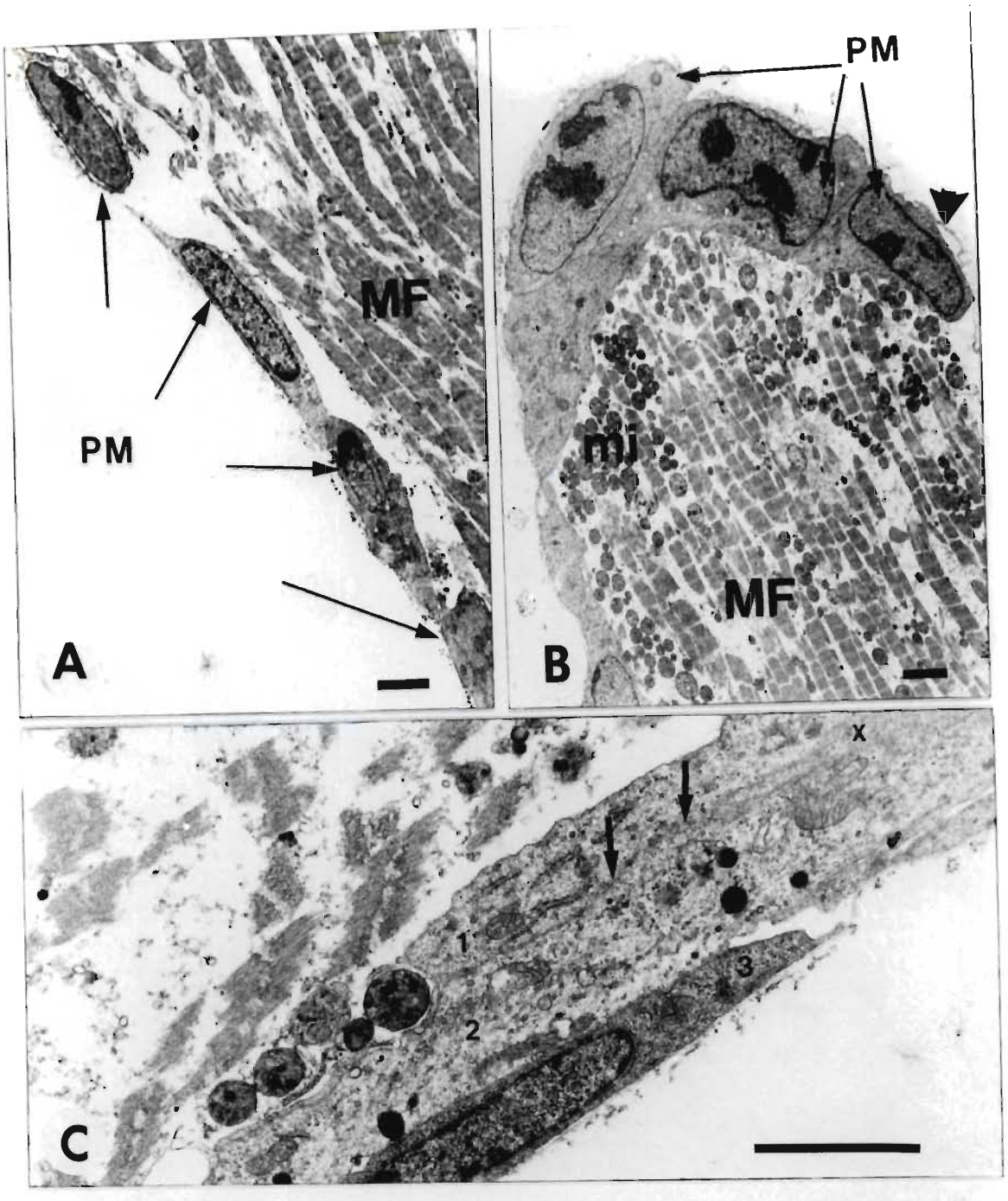


Figure 16. Electron micrographs of hamster explants, 3 to 5 days incubation showing increasing numbers of sublaminar cells and fusion. *A:* chains of sublaminar cells at various stages of development along a myofibre. *B:* a cluster of presumptive myoblasts at the end of a myofibre. Mitochondria increased in numbers in many myofibres. *C:* shows alignment of 3 cells, and fusion of myoblasts 1 and 2. MF, parent myofibre; mi, mitochondria; arrowhead, basement membrane; long arrows, presumptive myoblasts; short arrows, fusion between myoblasts; X, area between cells 1 and 2 where fusion appears complete. Bar = 2 μm.

microvesicles (Fig. 15*A*) which presumably were the forerunners of developing sarcoplasmic reticulum (Ishikawa, 1968). Some of these microvesicles were also considered to be pinocytic vesicles by others (Teravainen, 1970; Wakayama and Schotland, 1979; Campion, 1984).

As incubation progressed, the number of presumptive myoblasts within the myofibres increased. The increase in the number of cells was more rapid in the hamster than in the human explants. However, the large number of cells produced in both the muscles formed chains (Figs. 16*A* and 17*A*) of cells along the subsarcolemmal regions of the myofibres. In cross sections, the cells formed rings around the myofibres (Fig. 17*B*). Clusters of cells forming a cap or cone formation were common at the distal ends of many of the myofibres (Figs. 16*B* and 17*C*). These cells, lying adjacent to each other invariably displayed closely applied junctions (Figs 13*C*, 16*C* and 17*C*) or areas of membrane associations called fusion plaques (Trelstad et al., 1967). Apart from seeing fusion plaques between adjacent membranes, areas where fusion between the two cells was complete were also seen. However, at times, the different cytoplasm belonging to each of the cells undergoing fusion were recognized by the mosaic appearance of the fused cytoplasm. The mosaic appearance was due to varying electron densities of the cytoplasm of the different cells. Fusion was also said to take place by developing 'outpouching's (Mendell et al., 1972) and by cytoplasmic projections of different cells being engulfed one by the other (Shimada, 1971). The fusion between the cells led to the formation of multinucleate myotubes. In a number of myoblasts (Figs. 18*A* and 21*A*) and multinucleate myotubes (Figs. 18*B*, 19*B*, 19*C* and 22*B*), developing myofibrils with clear Z line development signalled the myogenic status of these cells. In cross sections of these myotubes, arrays of developing myofilaments with the

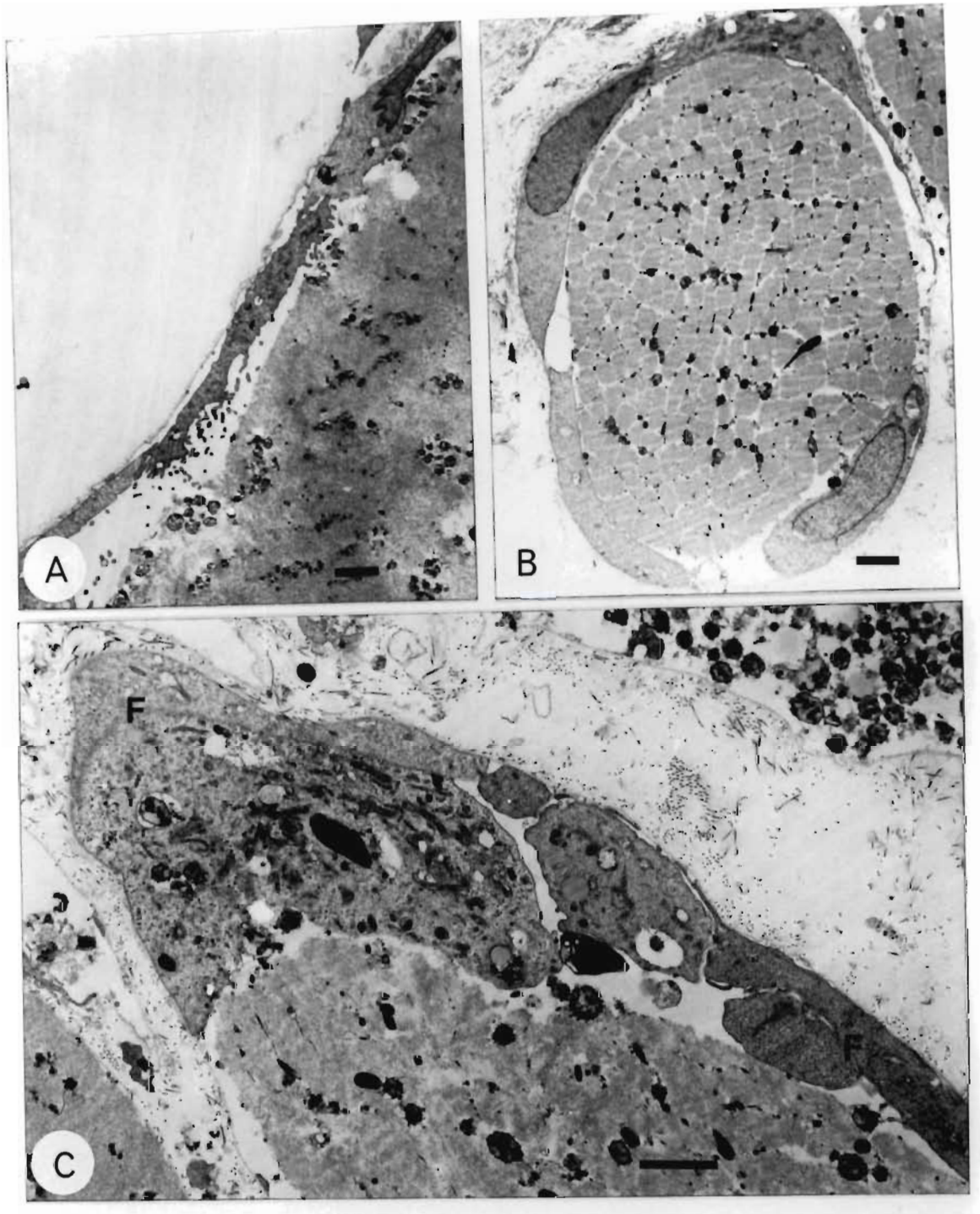


Figure 17. Electron micrographs of chains and clusters of young cells in human explants after 7 days incubation. *A:* Chain of sublaminar cells along the length of degenerate parent myofibre. *B:* Cross sectional view of young cells around the parent myofibre. *C:* An aggregation of cells at the end of a parent myofibre. Fusion (F) has begun between some of the cells to form myotubes. The cell capping the end of the myofibre has morphological appearance of cells, probably derived from cytoplasmic organelles, as described for the hamster. Mitochondrial aggregation is seen in the neighbouring myofibre in the top corner. Bar = 2 μm.

thick and thin filament arrangements were distinct. The presence of developing myofibrils in myoblasts and myotubes was well documented (Carlson, 1973; Mastaglia and Walton, 1971; Tautu and Jasmin, 1982; Jasmin et al., 1984a).

In the current study, the myotubes developed myofibrils up to a point and then began to exhibit degenerative features. Fully formed young myofibres with a full complement of myofibrils were not observed. In hamster muscle, the cut off point of further development of myotubes was between 5 and 7 days of incubation. It was difficult to deduce this in the human muscle because the general development was slow and perhaps the 10 days of incubation was too short.

The possible reason for the myotube not achieving full maturity to the status of myofibres was most likely due to the fact that the, ***in vivo***, situation was different from the, ***in vitro***, conditions in that nerve growth factors and other necessary humoral factors such as hormones were not present in the culture conditions. According to Carlson (1979), the morphology of the regenerates was usually imperfect and that the gross morphogenesis, internal architecture and quality of the regenerate depended on physical factors and the functional environment. Cytotoxic effect produced by degenerating fibres and the absence of trophic effects of the nerve (Ontell, 1975) as well as the absence of neurally evoked electrical activity (Ashby et al., 1993) were also suggested as possible reasons for failure to achieve complete regeneration.

During the later incubation period many new cell forms appeared in the myofibres and the possible derivation of these cells will be discussed later in this chapter.

5.5.7 Myoblasts from connective tissue elements

Although earlier workers (Adams et al., 1962) believed that muscle restoration was always the result of proliferation of subsarcolemmal nuclei and that connective tissue elements did not participate in the process of regeneration, Sloper and Partridge (1980) expressed the possibility of connective tissue cells contributing precursor cells for muscle regeneration.

Whilst identification between fibroblasts and myoblasts in culture were considered not possible by Witkowski (1977), it was suggested by Lipton (1977) that fibroblasts could be differentiated from myoblasts by the presence of extensive elaboration of endoplasmic reticulum and multiple Golgi complexes. It was also expressed by Lipton (1977) that in less than optimal environment, myoblast morphological differentiation may be modulated or reversibly altered.

Ontell (1977) reported the presence of mast cells in the sublaminar area together with satellite cells of neonatal rat muscle, and the part played by the mast cells was unknown. Cells with long finger-like cytoplasmic projections observed in frog muscle were regarded as invasive cells (Maruenda and Franzini- Armstrong, 1978) which were assumed to be monocytes which slowly differentiated to macrophages (Franzini-Armstrong, 1979).

There were other suggestions made that myoblasts were capable of behaving as phagocytes (Gilbert and Hazard, 1965; Carlson, 1973; Garfield et al., 1975; Yarom et al., 1976). Gilbert and Hazard (1965), from observations in dystrophic muscle biopsies, suggested that myoblasts

assisted in the dissolution of necrotic material during muscle degeneration and regeneration. In a gold tracer study (Yarom et al., 1976), myoblasts and even myotubes in regenerating extraoccular muscles of rabbits were shown to take up the gold particles, and were, therefore, said to behave as phagocytes. Mastaglia and Walton (1971) stated that the characteristics of some of the cells seen in polymyositis having cytoplasmic inclusions and autophagic vacuoles made differentiation between myoblasts and macrophages extremely difficult.

Reznik (1969) emphasized that the only mononucleated cells that contained myofibrils were clearly macrophages which phagocytized necrotic muscle fibre fragments, but went on to state that some macrophagic cells were derived from necrotic myofibres (Reznik, 1969). Trupin et al. (1979) made a strong case that many of the sublaminal fusiform cells considered by other researchers as myoblasts were erroneous and that most of these fusiform cells observed were phagocytes. Their study involved implants of frogs and rats. Some implants were treated with carbon ink particles and others were lyophilized before being placed inside the experimental animals. Although they felt that the distinction between the early satellite cells and the macrophages was not well defined, their experimental results led to the conclusion that the traditional criteria of cell shape and location used to identify cells during muscle regeneration were unreliable, and that the undifferentiated invading macrophages were able to mimic the appearance of the early endogenous myogenic cells.

Active degeneration and phagocytosis of muscle fibres and regeneration activity were shown to be wide spread in the clinical stages of muscular dystrophy (Hudgson et al., 1967) and in all forms of dystrophies (Pearce

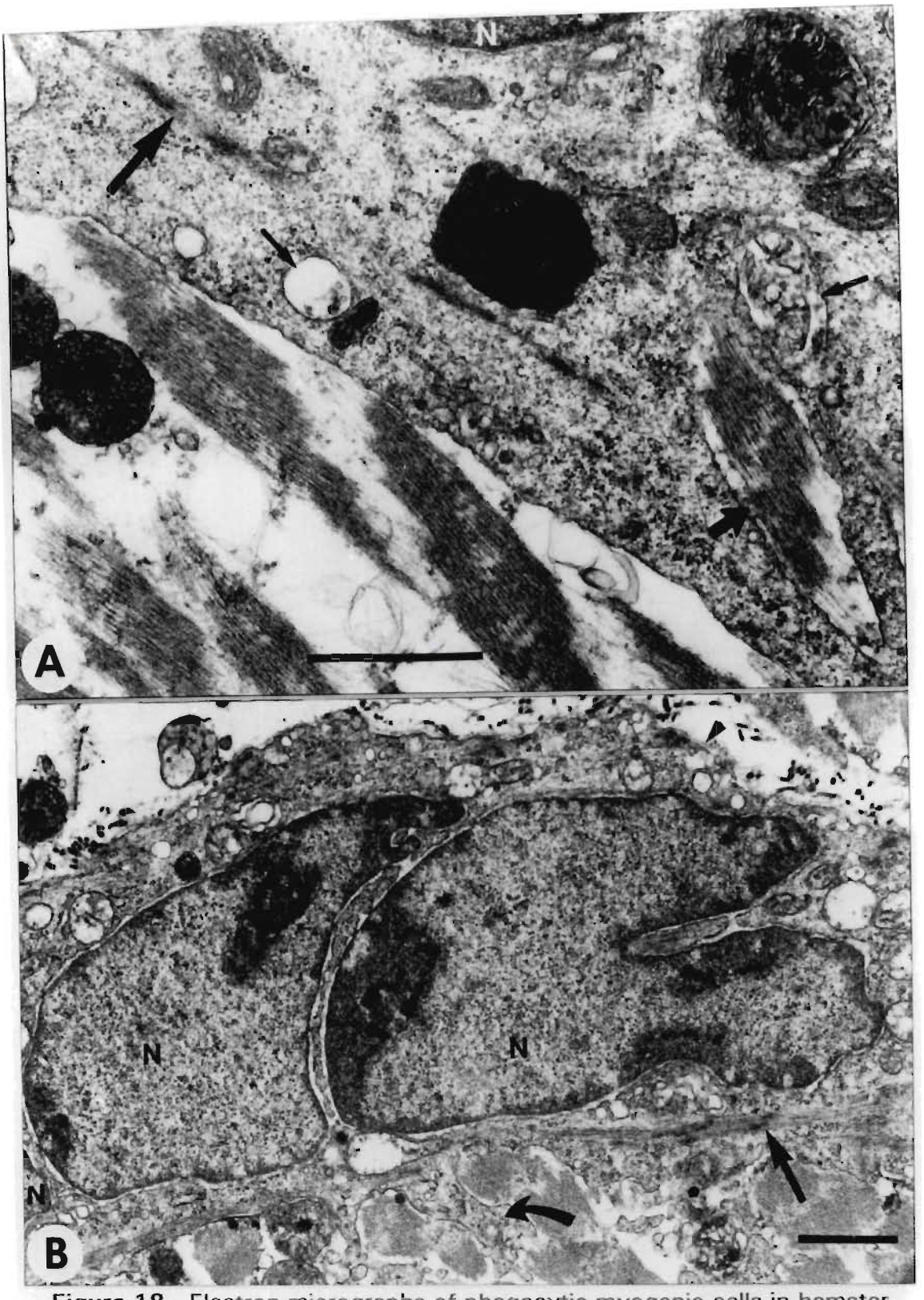


Figure 18. Electron micrographs of phagocytic myogenic cells in hamster explants, incubation day 4. *A:* part of myoblast mimicking a phagocyte having phagosomal like bodies containing degenerate fibrillar elements of the parent myofibre (short arrow), and autophagic vacuoles (thin arrows). *B:* Part of a multinuleate myotube with cytoplasmic projections (curved arrow) like that of phagocytes. Distinct developing myofibrils (long arrow) in the cytoplasm of the cells confirm their myogenic status. Arrowhead, basement membrane; N, nucleus. Bar = 1 μm.

and Walton, 1962). Studies on bupivacaine treated rat muscle (Bradley, 1979) showed that regeneration in myofibres occurred side by side with necrosis and phagocytosis after 4 days followed by extensive muscle fibre regeneration by the 6th day.

In the light of the above mentioned findings in literature on the question of phagocytic cells and myoblasts behaving as phagocytes in regenerating and pathological muscle, observations regarding the above issue were made in the current study. The hamster and human muscle explants showed that the early stages of regeneration were marked by an increase in the number of sublaminar cells which were invariably fusiform cells. As incubation time increased, these cells increased in girth and developed long tails of cytoplasm. Some sublaminar cells (Fig, 19*A* and appendix III) developed cytoplasmic projections or pseudopodia and the number of these cells showed an obvious increase with incubation time. There was no evidence to suggest that these cells entered the fibre from interstitial areas.

It was shown in other studies (Bischoff, 1979) that the basement membrane was tough and remained intact in single fibre cultures so much so that the cells that proliferated within the myofibre tube were contained inside and did not migrate to the outside. It is expected that it would be just as difficult for macrophages or phagocytes from outside to enter the myofibre. Seeing that the outside elements were also minimized by the explant technique, it is reasonable to suggest that the many sublaminar cells with pseudopodia are a stage in the development of myoblasts, and not invading monocytes which later developed into macrophages as suggested by Franzini-Armstrong (1979). Mastaglia and Walton (1971), in their study of pathological tissue, stated that elongated mononucleated cells were the earliest findings of regeneration, prior to the arrival of macrophages. Perhaps the macrophages

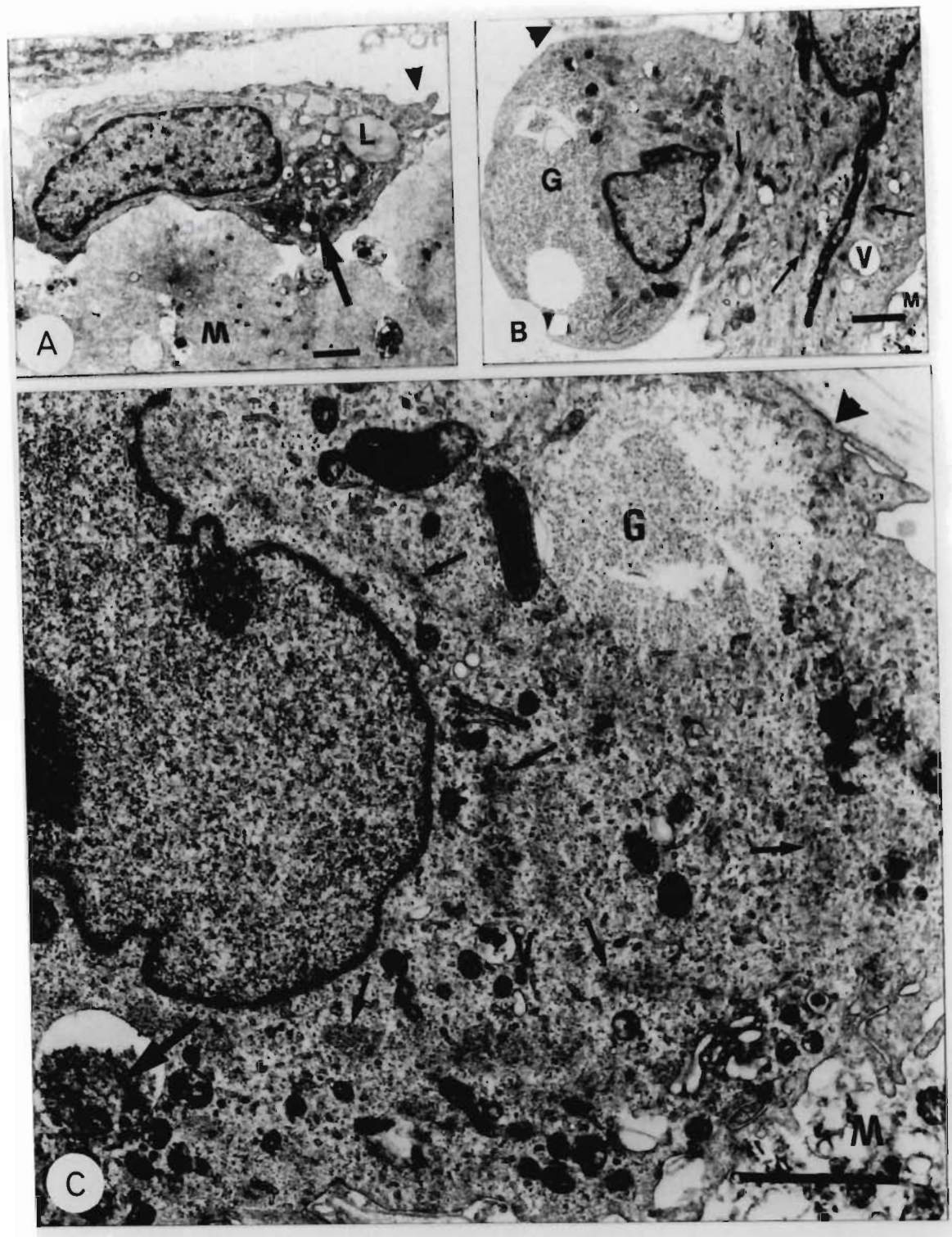


Figure 19. Electron micrographs of human explants showing cells phagocytic in appearance, 3 to 6 days of incubation. *A:* Sublaminar cell with lysosomes (L), extensive endoplasmic reticulum and autophagic vacuoles or phagosomes (arrow), and is of unknown identity. *B* & *C:* Myogenic cells or myotubes with morphological feature of phagocytes. V, vacuoles; G, glycogen body; M, myofibre; small arrows, developing myofibrils; large arrow, autophagic vacuoles; arrowhead, basement membrane; All of them have cytoplasmic projections similar to phagocytes. Bar = 1 μm.

which appear after the elongated mononucleated cells, as described by Mastaglia and Walton, are another stage in the development of myoblasts, and is complimentary to the findings of the present study.

Also, the sublaminar cells (Trupin et al., 1979; Mastaglia and Walton, 1971), containing lysosomes, autophagic vacuoles and phagosomes in their cytoplasm, are not necessarily phagocytes or macrophages according to the findings of the current study. Figures 18 and 19 show cells with morphological characteristics of phagocytes. Some of the phagocytic characteristics are attributed to the presence of degenerate myofibrils of the parent myofibre in phagosomes, autophagic vacuoles, large glycogen bodies, dilated endoplasmic reticulum, cytoplasmic inclusions and cytoplasmic projections. At the same time, the cytoplasm of some of these cells (Figs. 18*A*, 18*B*, 19*B* and 19*C*) contains developing or primitive myofibrils. The presence of the developing myofibrils indicated clearly that these cells are myogenic and that they were myotubes which could easily be mistaken for a phagocyte without careful scrutiny.

Most binucleate and multinucleate myotubes (Fig. 18*B*, 19*B* and 22*B*) with developing myofibrils may well be mistaken for polymorphic leucocytes. Furthermore, the early myofibrillar assemblies seen in the cytoplasm (Figs. 18*B*, 19*C* and 22*C*) were unmistakeable. Many of these phagocytic-appearing myotubes had developing myofibrils which revealed electron dense zones, characteristic of Z line material and similar to those described by Mastaglia and Walton (1971).

In the current study, it is shown that the cells with myogenic potential, found in a sublaminar position during regeneration of muscle, may have

variable morphology appearing as fusiform cells or as phagocytes. The morphological configuration of these sublaminar cells depended on the availability of space within the myofibre. A logical explanation for the early cells being fusiform would be that the myofibres at this stage still had intact myofibrillar material so that myoblasts could only develop sandwiched between the basement membrane and the myofibrils. As degeneration and fragmentation of myofibrils occurred, space became available within the myofibre, allowing the myoblasts to develop cytoplasmic projections or pseudopodia. During the course of their development to myotubes, it appears as if the myoblasts engulf or phagocytize cytoplasmic elements from the degenerating parent myofibre, not so much to remove debris, but to use them as a source of nutrients for their growth. Therefore, in doing this, their morphology resembles that of true phagocytes, but they are myogenic cells. Mastaglia and Walton (1971) also support this by having suggested that regenerating cells may "*actually reutilise breakdown products of necrotic sarcoplasm*". Perhaps the observation of similar cells in early regeneration studies led to the assumption that phagocytes and other connective tissue cells may contribute new cells during muscle regeneration (Sloper and Partridge, 1980).

Earlier regeneration studies (Snow, 1979; Allbrook, 1962; Baker and Poindextor, 1991) were performed mainly on minced or injured muscle which was left inside the experimental animals. These, ***in vivo***, studies constituted a problem in that the injured myofibres rapidly underwent necrosis as a result of poor circulation due to blood clots. "Outside-muscle" influences (Askanas, 1979) such as migrating phagocytes and fibroblasts also made clear identification between myoblasts and other sublaminar cells in the, ***in vivo***, studies difficult. Therefore the sublaminar cells reported in earlier work could well be myogenic cells or phagocytes.

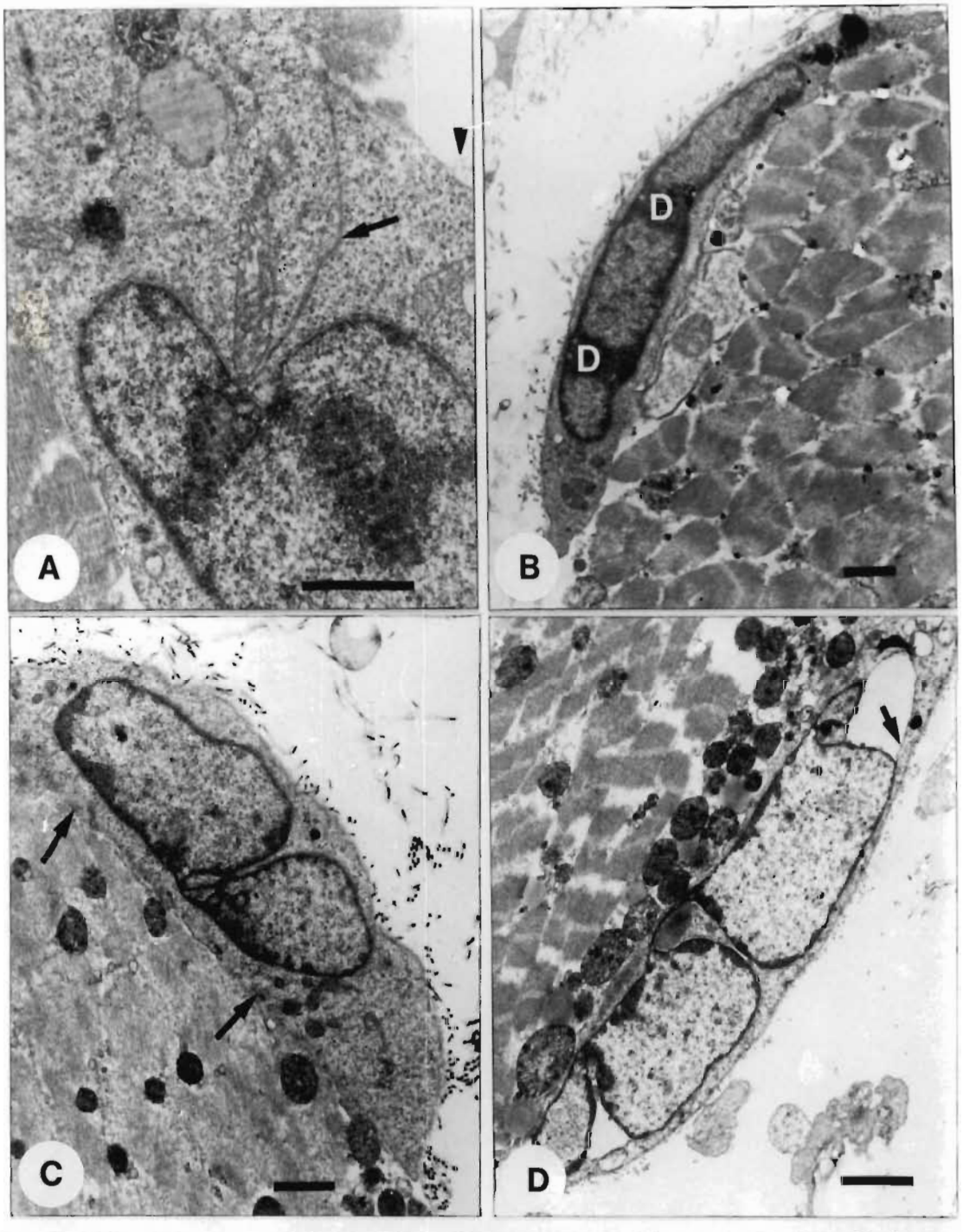


Figure 20. Electron micrographs of hamster myoblast nuclei 3 to 8 days of incubation revealing possible amitotic division. *A:* Arrow points to membrane invagination leading internally to the nucleus, electron dense with vesicles at this point. A mitochondrion is at this point. *B:* Nucleus with electron dense zones, D with slight constriction at these points. *C:* A bilobed nucleus linked by a thin band of nuclear material. Developing myofibrils (arrows) are present in the cytoplasm. *D:* Multilobed nucleus linked by thin bands of nuclear material. Two smaller lobes at either poles appear as 'nuclear buds', all this appearing within a vacuole caused by the separation of the outer nuclear membrane (small arrow). Bar = 1 μm.

5.5.8 Myonucleation: possible morphological representations of amitotic division of nuclei in myoblastic cells

During embryonic myogenesis, myonucleation or multinucleation or the increase in the number of myonuclei of muscle fibres was achieved by fusion of myoblasts (Speidel, 1938; Fischman, 1979). During post natal life, myonucleation was attributed to satellite cells proliferating mitotically and subsequently fusing with the myofibre (McConnachie et al., 1964; Moss and Leblond, 1970; Allbrook et al., 1971; Snow, 1977a/1979). Mitotic studies revealed that divisions in this way occurred in free undifferentiated myoblasts and not in formed myofibres (Gilbert and Hazard, 1965; Shafiq et al., 1968). Cells found fusing at the end regions of young myofibres of skeletal muscle in mice indicated a possible way in which nuclei were added to growing fibres (Williams and Goldspink, 1971).

Proliferation of cells sprouting from cultured myofibres (Bischoff, 1975), explants (Jasmin et al., 1984a&b) and from dissociated mononucleated cells (Yasin et al., 1977) supplied ample proof that myoblastic mononucleated cells (commonly referred to as satellite cells) underwent mitotic activity. On the other hand, Carlson (1973) and Reznik (1976) in their review, reported the absence of mitotic division in sarcoblasts (myoblasts) or multinucleated myotubes. Mitotic division of sublaminar cells were not seen in the current study as well. Furthermore, if mitotic division of cells were not recognized during the EM study, with the large number of myofibres inspected, at least some of the sublaminar cells ought to be found in pairs. This was not seen either in the current study. There is no doubt that the myoblastic cells, sprouting from explants or those cultured as enzymatically dissociated mononucleated cells, were capable of mitotic division. But why they are not

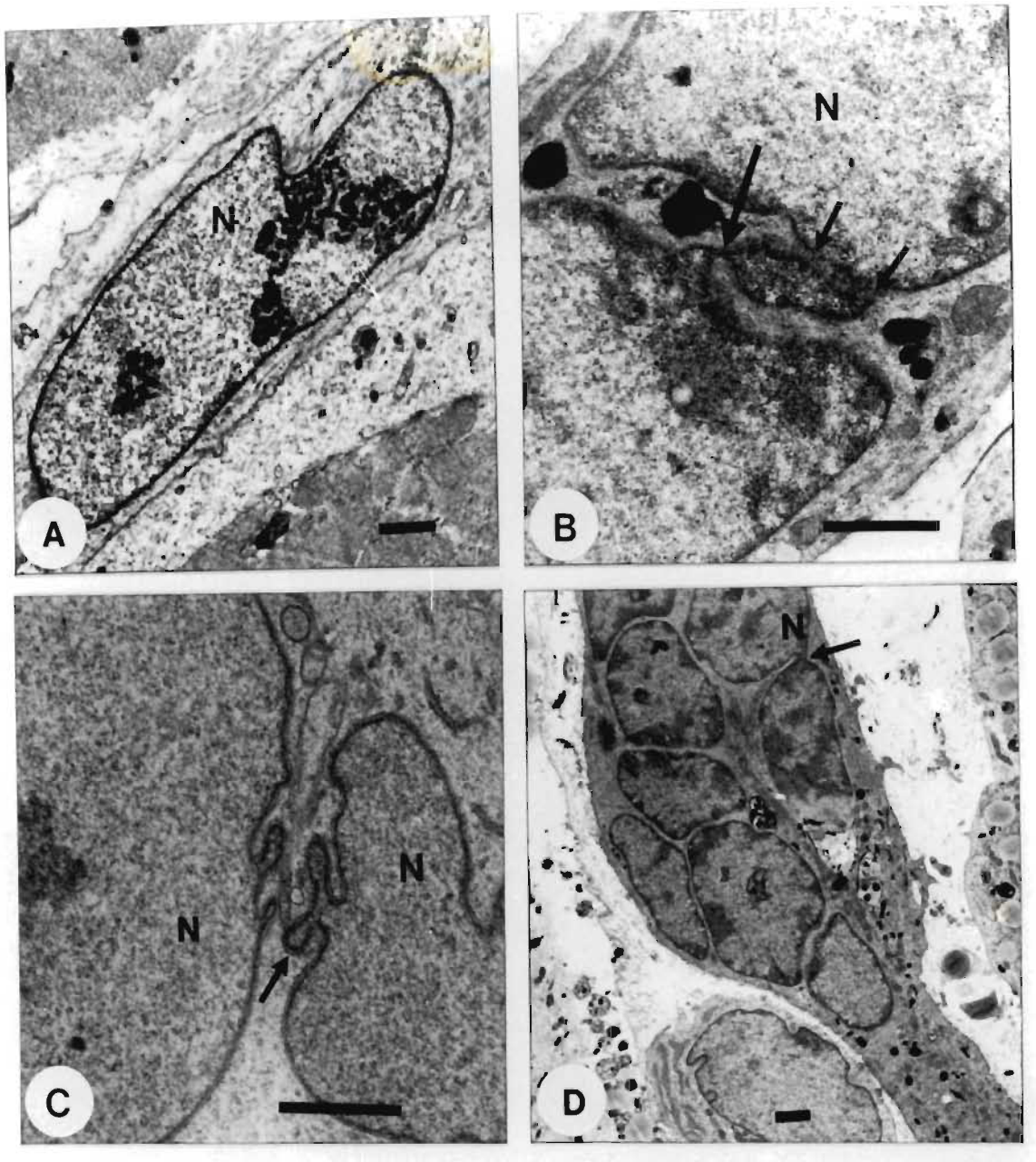


Figure 21. Electron micrographs of myogenic cells, from human explants, presenting morphological evidence for amitotic division. *A:* Myoblast nucleus on day 1 of incubation, with electron dense chromatin material leading from a cleft in the nucleus and branching into 2 different columns which perhaps mark the zone of future breaks in the nucleus. *B:* A seemingly bilobed, myoblast nucleus (7th day). The band of nuclear material between the lobes shows constriction (long arrow) and apparent separation (small arrows) from the larger lobes. *C:* Myotube with 2 nuclei seen on the 5th day. Both nuclei have 2 membrane bound projections (arrow) on opposed sides providing possible evidence that these nuclei were linked together. *D:* Part of multinucleate myotube located in interstitial space on 7th day. Nucleus labelled N is linked to another nucleus by a thin nuclear band (arrow) showing possible mechanism of myonucleation within myotubes. Bar = 1 μm.

observed in the myofibres of regenerates is unknown. A possible answer is that environmental factors may have a modulating influence on the nuclear activity.

Migration of satellite cells was another suggestion forwarded by some researchers for the increase in the number of cells. Satellite cells were assumed to migrate from damaged to undamaged areas of muscle (Mastaglia and Walton, 1971; Schultz et al., 1985; Hughes and Blau, 1990). Migration of myoblasts in injured muscle was thought to be due to chemotactic influence exerted by the injured myofibre (Watt et al., 1994). However, poor migration of injected donor myoblasts was said to be the cause of poor positive dystrophin response in myofibres of recipients undergoing myoblast transplant trials (Satoh et al., 1993). This somewhat negates the migration story of myoblasts. Tremblay et al. (1993) transplanted cultured myoblasts obtained from an asymptomatic monozygotic twin to the other who was a symptomatic carrier of DMD. Even though there were no immune problems, with the donor and the recipient being monozygotic twins, there was a small beneficial effect of the transplant with only slight increase in dystrophin positive fibres, and the poor result was attributed to possible low level spontaneous muscle regeneration. On the other hand, the failure of the above transplant could also be attributed to the fact that the myoblasts did not migrate into the neighbouring myofibres at the injection sites and the slight increase in dystrophin positive fibres could be due to the new fibres formed from only the myoblasts transplanted. Furthermore, Bischoff (1979) emphasized in his study of cultured fibres that the basal laminar tube was resilient so much so that the many cells that stacked the inside of the myofibre were not able to get out of the myotube. If they were not able to get out, it was not unreasonable to assume that other cells would be unable

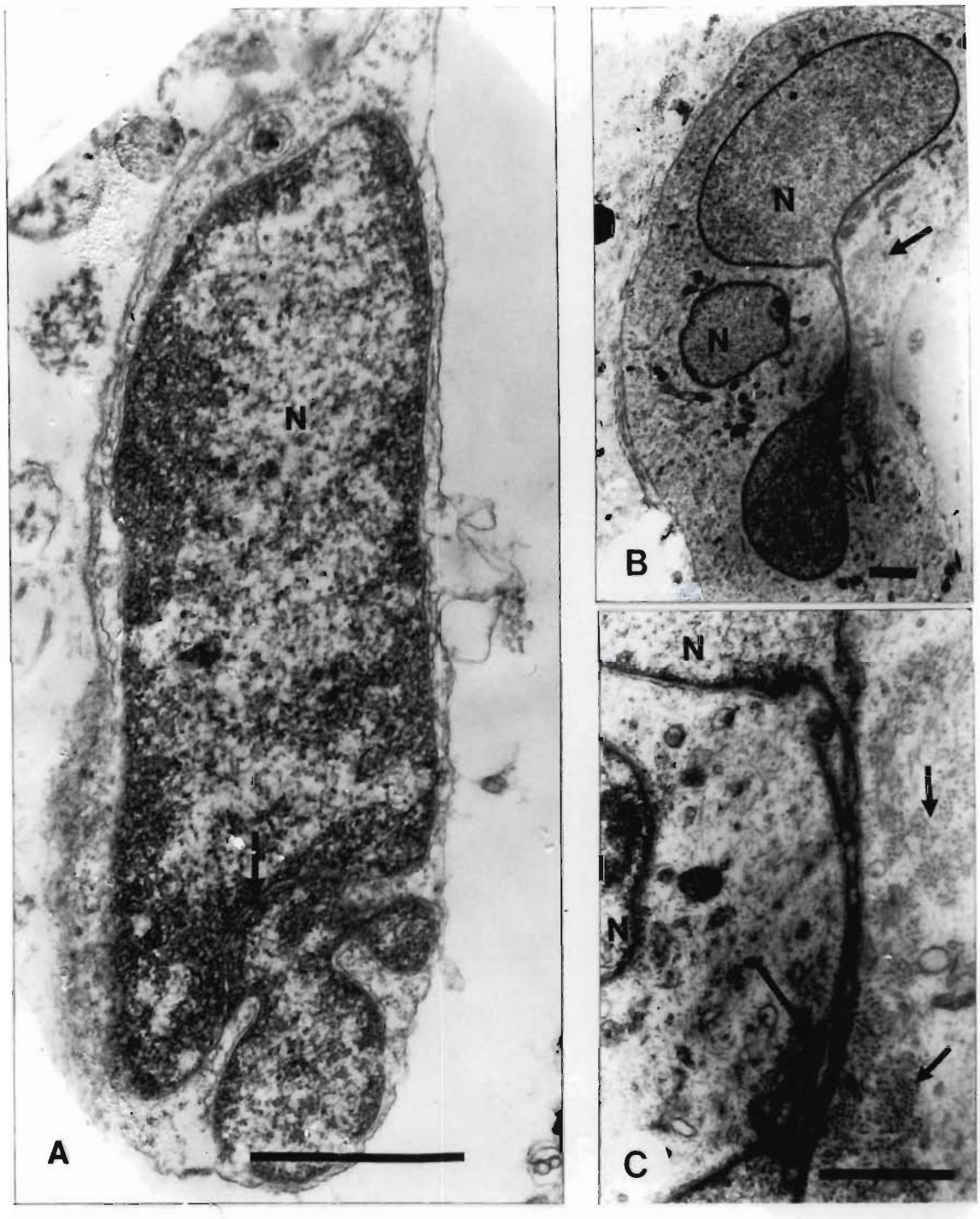


Figure 22. Electron micrographs of myogenic cells from human explants revealing evidence for possible amitotic nuclear division. *A:* a myoblastic cell within a degenerate myofibre on the 5th day. A deep, membrane bound invagination at the lower end (arrow) mark lines of possible segmentation of the nucleus (N). *B & C:* parts of the same myotube containing arrays of developing myofibrils (arrows) in cross section, a bilobed nucleus linked by a ribbon of nuclear material, and another nucleus. Incubation day 8. *C:* large arrow shows an extended nuclear tail that was possibly linked to the independent nucleus. Bar = 1 μm.

to get into the myofibre unless there was a lesion in the basement membrane. In the current study, most of the basement membranes of myofibres were intact, except at portions that were cut during the preparation of the explants.

In the present EM study, nuclei of sublaminal cells in both the hamster and human muscle explants revealed morphological outlines of possible amitotic division. In the human explants, some of these possible amitotic divisions were observed even in the control specimens that were not cultured. As explained before, regenerative features prevailing in the control specimens were perhaps due to drug and anaesthetic influences on the muscle of the patients. However, similar patterns of possible amitotic division were observed in both the hamster and the human muscle explants for most part of the incubation period. These division patterns of budding, splitting or segmentation, were generally observed in the more developed sublaminar cells with developing myofibrillar elements in their cytoplasm.

Early signs of segmentation were apparently denoted by electron dense zones of chromatin either stretching across the nucleus (Fig. 20*B*) or beginning at clefts in the nucleus (Figs. 20*A* and 21*A*) and diverging in two directions to perhaps break the nucleus into three parts. The two latter figures, showing more or less identical patterns, co-incidently came from hamster and human muscle, respectively. A deep membrane invagination (Fig. 20*A*), which probably represents the early sarcoplasmic reticulum, leads from the surface of the plasmalemma to the electron dense zone at the cleft. A longitudinal mitochondrion meeting at this point perhaps serves the energy requirement at this point. Microvesicles similar to those seen associated with the electron dense bodies opposed to Z lines were seen in

the electron dense zone of this nucleus. They are, perhaps, contributions from the fine sarcoplasmic reticulum to provide for future membranes.

In some myoblasts, two large lobes of the nucleus were linked by a very thin band of nuclear material (Figs. 20*C* and 21*B*). The band of nuclear material is constricted at one lobe and is apparently separated from the other lobe in Figure 21*B*. The morphological outline presented by this bilobed nucleus predicts 3 possible nuclei in the cell, in the future. The binucleate morphology shown in another myotube (Fig. 22*B*) probably was derived by separation of the smaller unattached nucleus from the larger bilobed nucleus held together by thin band of nuclear material. A small extended tail of nuclear material shown on higher magnification (Fig. 22*C*) probably represents the previous attachment point of the bilobed nucleus with the unattached nucleus. The electron dense area, seen in the unattached nucleus and being opposed to the nuclear band, perhaps represents its previous point of attachment. In another instance (Fig. 21*C*), 2 nuclei reveal 2 membrane lined projections on opposed sides providing possible evidence that this was a bilobed nucleus attached at these membrane projections. The presence of sarcoplasmic reticulum-like membranes and mitochondria between these 2 nuclei denote activity in this area.

Multilobed nuclei in myoblasts of hamsters were commonly observed during the latter part of the incubation period (Fig. 20*D*). These multilobes were often observed within vacuolations caused by the extended or distended outer membranes of the nucleus. The lobes again were attached by thin bands of nuclear material. The lobes at either poles of the nucleus appeared as though they were nuclear buds. The large number of mitochondria, observed below the cell, are there, perhaps, to provide the energy requirements for the activity within the cell.

Possible segmentation or splitting of nuclei of myoblasts were also observed. Deep membrane bound invaginations observed in some nuclei of myoblasts (Fig. 22*A*) possibly signalled segmentation or splitting of the nucleus. These nuclei were often electron dense with scattered heterochromatin resembling the morphology of satellite cells. Mutlinucleate myotubes were seen to develop in the interstitial spaces between the myofibres in the explants during the late incubation period. In one such myotube (Fig. 21*D*), two of the many nuclei within it were attached to each other. This observation probably answers how myonucleation takes place in myotubes once the nucleus becomes post mitotic. To date, it is not known how myonucleation takes place within the myotubes.

Godman (1957) in his study on regeneration and differentiation of mammalian striated muscle using rabbits, suggested that amitotic nuclear division was responsible for myonucleation. According to Cooper and Konigsberg (1961), the assumption that nuclei with dumbbell shapes and those with clefts were signs of amitotic division, was erroneous. Nuclei, in their view, assumed these shapes as they supposedly moved through, perhaps, restricted areas in the cytoplasm. However, the problem with the above two studies was that the conclusion was arrived at with the light microscope, with which it was difficult to observe such fine detailed events.

Assuming Cooper and Konigsberg (1961) were correct in their assumption, that assumption would not apply here as the nuclei were not restricted in their movement. If the cells had to move through restricted areas in the myofibre, then the cytoplasm of the cells would also assume similar shapes.

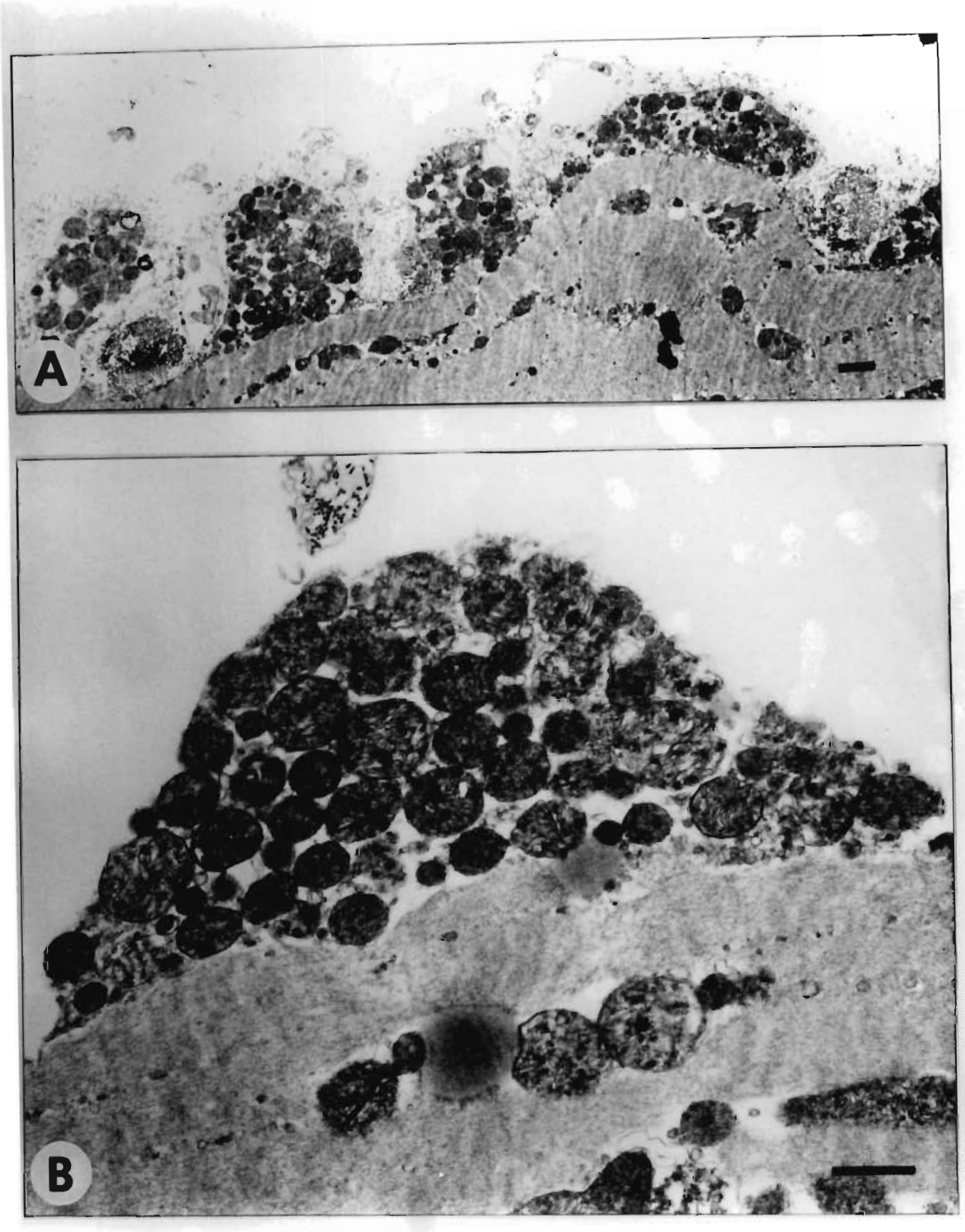


Figure 23. Electron micrographs of mitochondrial aggregations in hamster explants after 6 days incubation. *A:* Convoluted membranes of myofibres with clusters of mitochondria between it and the myofibrils, forming waves on the periphery of many myofibres in specimens from all animals. *B:* Single cluster of mitochondria with paracrystalline bars, dense osmiophilic bodies and some with vesicles. Mitochondria at deeper levels of the myofibre showed similar morphological changes. Bar = 1 μm.

However, viewing the electron micrographs presented, their is no sign that the cells or the nuclei in question suffered restrictions for space in the degenerating myofibres. Furthermore, in all the dissociated mononucleated cells viewed, as those shown in Figure 1, the nuclei did not exhibit any polymorphism at that early stage.

The conclusion from the polymorphic structures of nuclei described above was that these morphological patterns signified amitotic division of the nuclei of the myoblasts within the myofibre. This would explain why no mitotic figures were seen by many of the researchers. It was unfortunate that one can not do a time study on these myoblasts within the myofibres with cinephotography to capture the divisions of the nuclei "on the act."

5.5.9 An hypothesis for the generation of new cytoplasm, nuclei and cells

Most of the discussion that follows would seem to go against the dictum of biological science. Nevertheless, it is regarded as a duty of a research scientist to place on record whatever is observed during the course of research work undertaken and forward a possible explanations for such observations, however implausible they may sound. This is said because the following deliberations pivot on the generation of new cytoplasm and new nuclei apparently leading to the formation of "new generation cells." The evidence for all of this is presented by the apparent sequence of morphological structures, prevailing in the incubated muscle explants, captured in the electron micrographs.

However, the burden of making such announcements is lightened by the fact that another EM study (Lee, 1965), performed on rabbits and rat after prolonged denervation of muscle, describes cells which "*appeared to form*

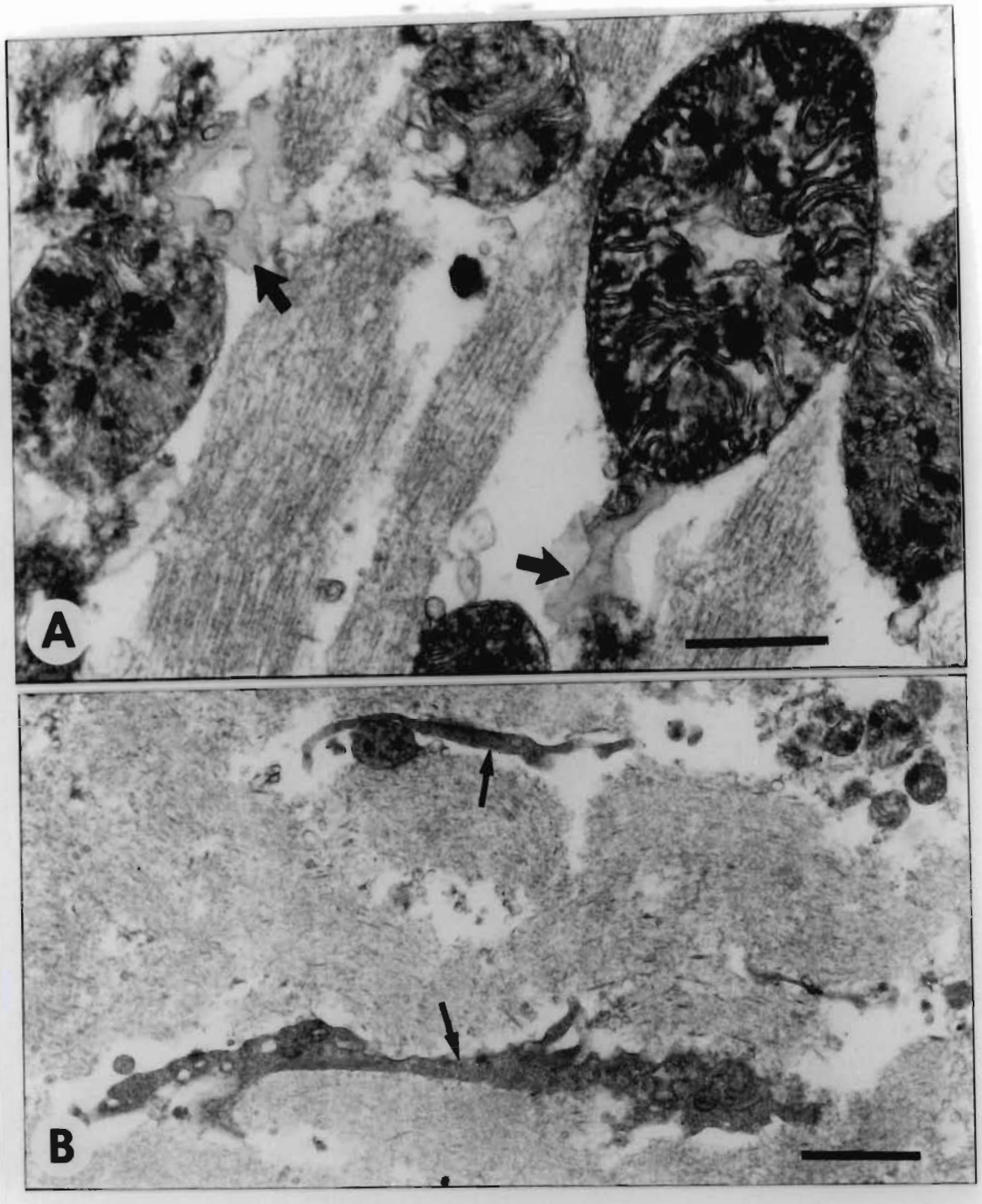


Figure 24. Electron micrographs of plasmic forms in hamster explants after 6 days incubation. *A:* Undifferentiated plasmic forms (arrows) associated with mitochondria in central regions of the degenerating myofibres in a number of explants. *B:* Strands of differentiated cytoplasmic forms (arrows) in the central regions of a degenerate myofibre. In all these cytoplasmic forms viewed, there was no evidence of any nuclear presence. One can only speculate that these cytoplasmic forms were derived from the plasmic forms described in *A*, perhaps by internal **'potocytosis'**.
Bar = 0,5 μm in *A* and 1 μm in *B*.

by the compressed sarcoplasmic reticulum that encompassed a portion of sarcoplasm with a nucleus" in the central areas of atrophic and degenerating myofibres. Lee went on to say that the mechanism of formation of the cells, "by enclosing and ejecting some intracellular structures" also occurred in other tissue cells and that this mechanism was called "*potocytosis*" by Meltzer in 1904.

5.5.9.1 Mitochondrial proliferation and aggregation

It was already mentioned that in any one animal or human series of explants, mitochondrial numbers increased with the incubation period. Although it was stated in literature (Sewry, 1985) that increased number of mitochondria and mitochondrial aggregations were sometimes observed in normal muscle, proliferation of the mitochondria forming waves of mitochondrial cluster under convolutions of the sarcolemma along the fibres in the muscle explants was phenomenal (Fig. 23). Clusters of mitochondria were also positioned at the terminals and in the intermyofibrillar spaces of many of the myofibres. This proliferation was more pronounced in the hamster than in the human muscle because regenerative changes were much slower in the human explants, as explained earlier in the discussion. However, proliferation of such clusters were seemed somehow to be replaced by cells as incubation time increased. Instead of seeing waves of mitochondria along the fibres, waves of cells seemed to have taken their place. Often many mitochondria were still associated with these cells (Fig. 28*A*). At this point it must be mentioned that sarcoplasmic elements were also amongst the mitochondrial aggregations, but their presence was overshadowed by the large number of mitochondria.

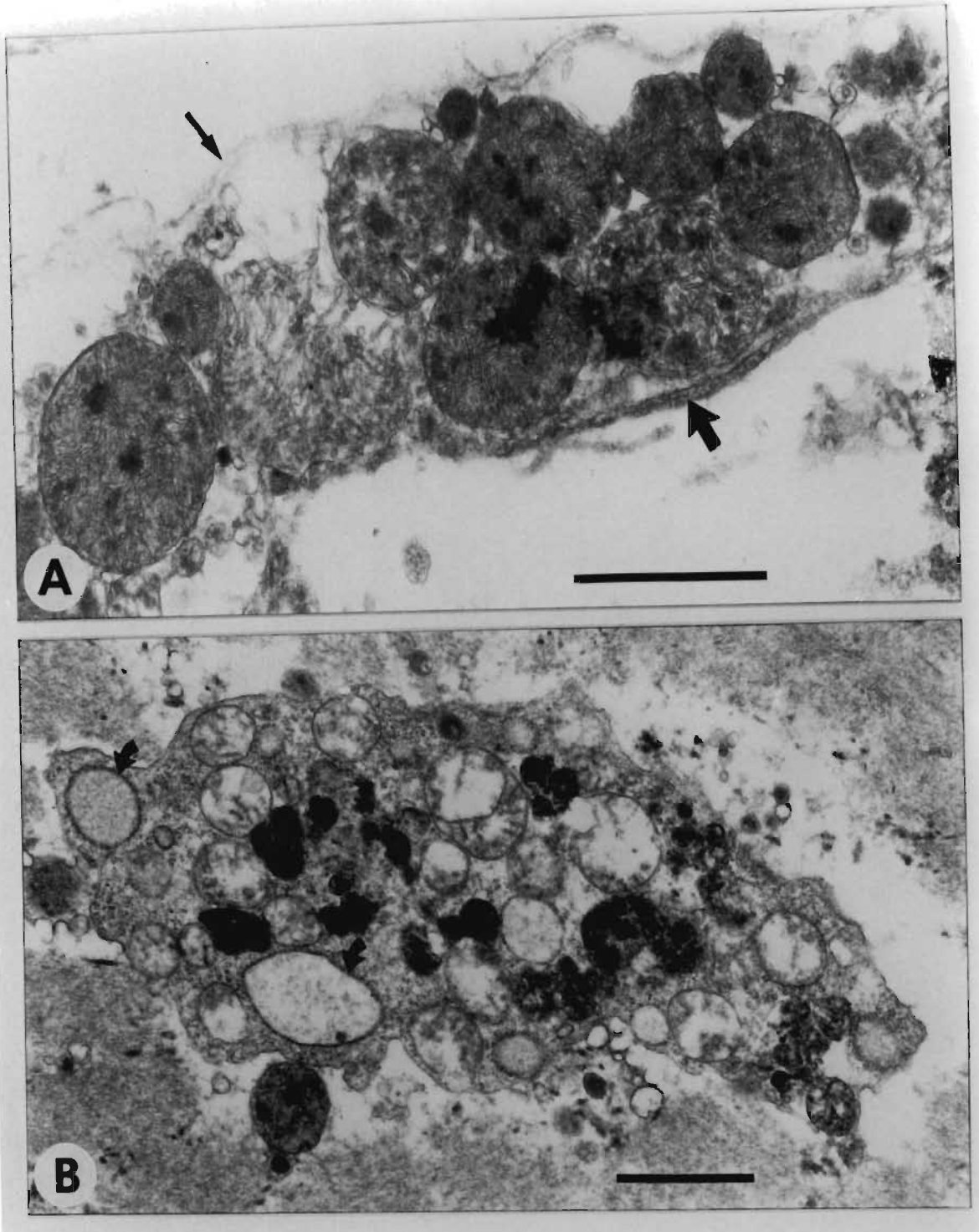


Figure 25. Electron micrographs of precursors of new cell generation in hamster explants, incubation day 6. ***A:*** Sublaminal cluster of mitochondria under the basement membrane (thin arrow) partially bordered by cytoplasmic strand in the lower aspect (thick arrow). ***B:*** A differentiated cytoplasmic formation in between the degenerate myofibrills containing dense cytoplasmic bodies, some similar to the electron dense mitochondria outside it, and dilated endoplasmic reticulum (arrows). Nuclei were not seen associated with these cytoplasmic forms. Bar = 1 μm.

An attempt will be made to explain how these cells are derived in the cultured explants from the many electron micrographs which captured events that provide a sequence of morphological changes.

5.5.9.2 "New generation cytoplasm"

In the central regions of many degenerate myofibres of hamster explants, electron dense mitochondria, with electron dense osmiophillic bodies were seen associated with undifferentiated hyaloplasmic forms (Fig. 24*A*). It appeared as though the plasmic material was exuding out of the mitochondria. Many of these mitochondria exhibited disruption and vacuolation in the intercrystal spaces. In the central regions of many other fibres, strands of slightly more differentiated cytoplasmic forms (Fig. 24*B*) with vacuoles and granular cytoplasm, without much other organelle structures were seen. Coincidentally, the electron micrographs of the undifferentiated plasmic and the differentiated cytoplasmic strands presented came from the same animal on two different days of incubation, one following the other. First impressions would be that these cytoplasmic strands belonged to cells whose nuclei were either above or below the plane of sectioning. Although serial sectioning was not carried out, it can be safely said that in the many cytoplasmic strands observed in the different degenerate myofibres, nuclei were not associated with them, nor were there many developed organelle structures in them, and the latter perhaps signified their immaturity.

In other instances cytoplasmic organelles, mainly mitochondria together with some vesicular structures, partially bordered by cytoplasmic strands (Fig. 25*A*), were observed in the subsarcolemmal regions of the myofibres. It

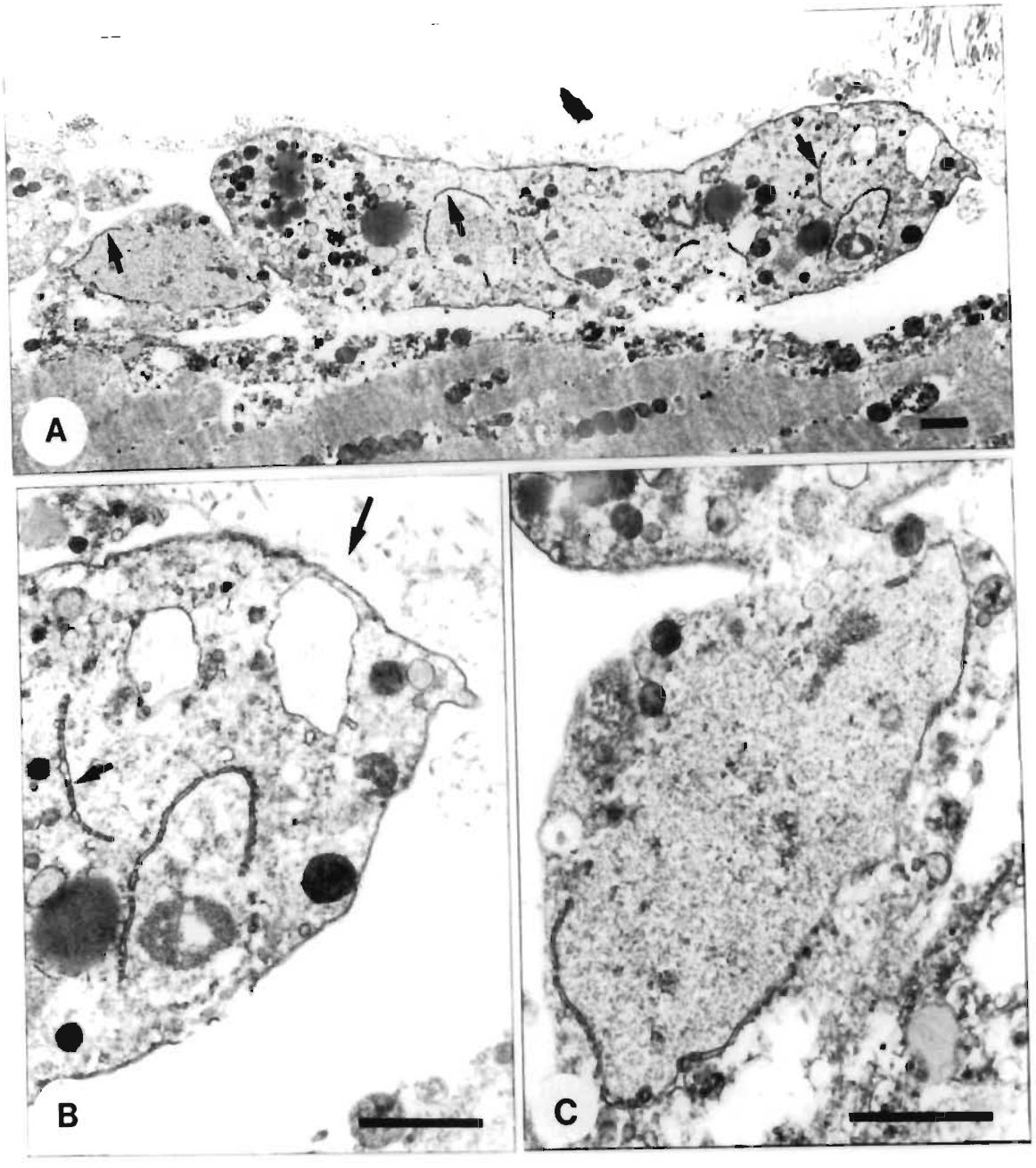


Figure 26. Electron micrographs of precursors of new cell generation in hamster explant after 6 days incubation. *A:* Differentiated cytoplasmic form in the sublaminal region, containing membrane structures (arrows) randomly distributed within. *B* & *C:* Sections of **A** clearly showing the double membrane structure of nuclear membranes. The granular plasmic formation within the partially formed membranes in *C* contain numerous inclusions. The picture presented leads one to speculate that these membranes mark the early formation of nucleus within cytoplasmic forms derived from cytoplasmic organelles, mainly the mitochondria and the sarcoplasmic reticulum, somewhat similar to the phenomenon, potocytosis. They perhaps also give a clue as to how inclusions find themselves inside nuclei in diseased tissue. Bar = 1 μm.

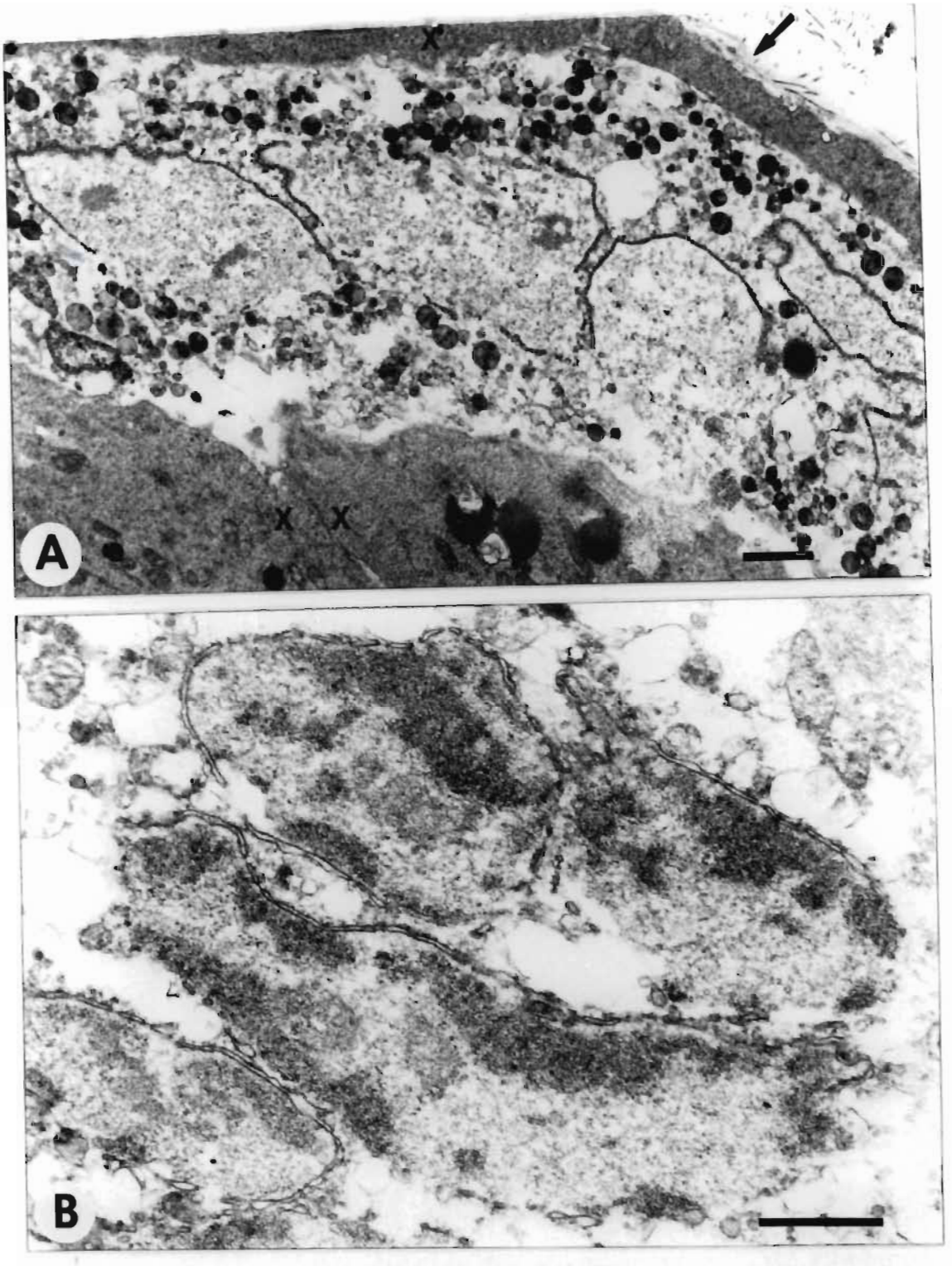


Figure 27. Electron micrographs showing generation of new nuclei in hamster explants after 6 days incubation. *A:* Terminal part of a degenerate myofibre with presumptive myoblasts (X) at different levels with dense granular cytoplasm. The vacuole between the cells contains dense aggregation of cytoplasmic organelles amidst which double membranes previously described are visible. *B:* Another explant revealing the same evidence of double membranes similar to nuclear membranes. The appearance of chromatin-like patches within the partially enclosed membranes testifies to the formation of "new generation nuclei" of future cells. Bar = 1 μm.

would appear that this cytoplasmic formation around the organelles led to the formation of the differentiated cytoplasmic masses without nuclei, identified in subsarcolemmal and central regions of the myofibres (Fig. 25*B*).

These cytoplasmic bodies had autophagic vacuoles, electron dense bodies similar to the electron dense mitochondria found outside them and dilated endoplasmic reticulum. Similar structures were described by Lee (1965) in a study on denervated muscle. Characteristic nuclei were not seen associated with all these cytoplasmic masses identified. It is suggested that these cytoplasmic masses, apparently formed in association with the sarcoplasmic organelles mainly the mitochondria by yet unknown mechanisms, represent "new generation cytoplasm".

5.5.9.3 "New generation nuclei"

In some of the differentiated cytoplasmic masses, doubled membrane structures were observed to be randomly distributed in the cytoplasm. Their random distribution and the distances separating them suggested that it was unlikely that they belonged to degenerate nuclei (Fig. 26*A*). These cytoplasmic structures were still attached or intimately associated with mitochondrial and other sarcoplasmic organelles. Higher magnification of these membrane fragments revealed a doubled nuclear membrane structure (Fig. 26*B*). In extensions of some of the cytoplasmic masses (Fig. 26*C*) membranes were observed to partially cordon off material similar to granular nucleoplasm with many inclusions. The same phenomenon of nuclear membrane forming around nucleoplasm like material was also observed in human muscle explants (Fig. 32*B*).

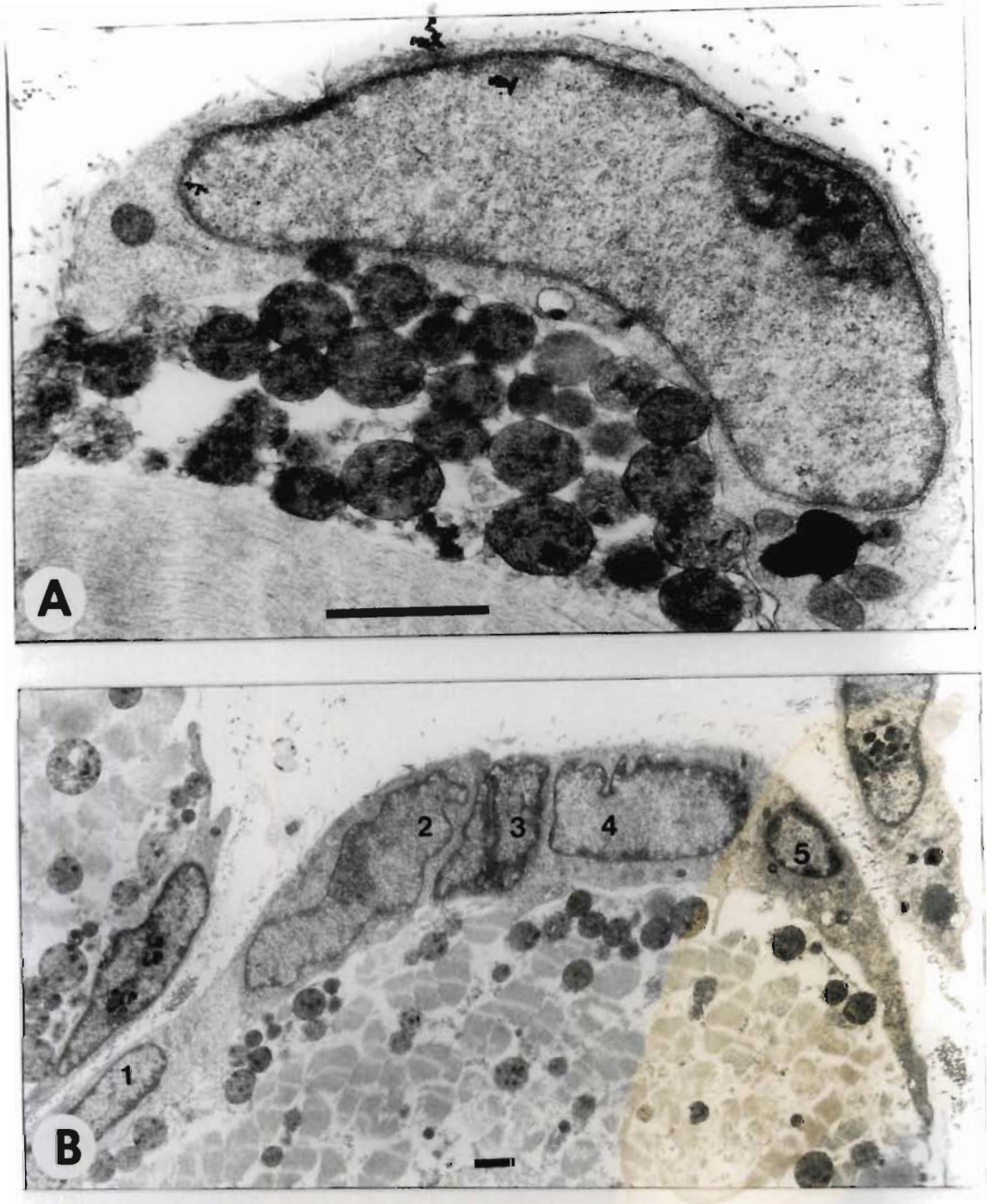


Figure 28. Electron micrographs of newly generated cells in hamster explants after 6 days incubation. *A:* Mononucleated cell found in areas of myofibres previously occupied by clusters of mitochondria and other organelles, the evidence prevailing below the new young cell. *B:* It is suggested that cells 1, 2, 3, 4 and 5 including those in the neighbouring myofibres were derived in the same way. Bar = 1 μm.

At the terminal parts of many myofibres, mitochondrial aggregations seemed to have been replaced by clusters of cells. At some terminals, large vacuoles (spaces), containing jagged cytoplasmic forms with dense aggregation of sarcoplasmic organelles, were formed by cells lying above and below them (Fig. 27*A*).

Again, large number of mitochondria were apparently the greater proportion of the sarcoplasmic organelles. Amidst this mass of sarcoplasmic organelles and jagged cytoplasm, membranous forms drew incomplete patterns of irregular nuclei. Higher magnification of similar membranes in another explant (Fig. 27*B*) showed the membrane structures to be similar to that of nuclear membranes. The appearance of chromatin-like patches within the partially enclosed membranes is suggestive of the formation of "new generation nuclei."

At first sight of these incomplete nuclei like structures, one may come to the conclusion that these were pictures of degenerate nuclei. That may be a correct assumption if there were multinucleate forms in the incubated explants preceding the observation of these incomplete nuclear structures. Therefore, in the absence of such aggregation of nuclei in earlier incubated explants it is reasonable to state that the incomplete nuclear forms herald the formation of multinuclei found at the terminals of many of the myofibres (Figs. 29). Furthermore, the irregular morphology of the multinuclei found in myotubes at the terminals show a similar irregular morphology as that of the incomplete forms of nuclei.

The original size of the aggregations of the sarcoplasmic organelles, which visibly comprised mainly of mitochondria, would seem to indicate the

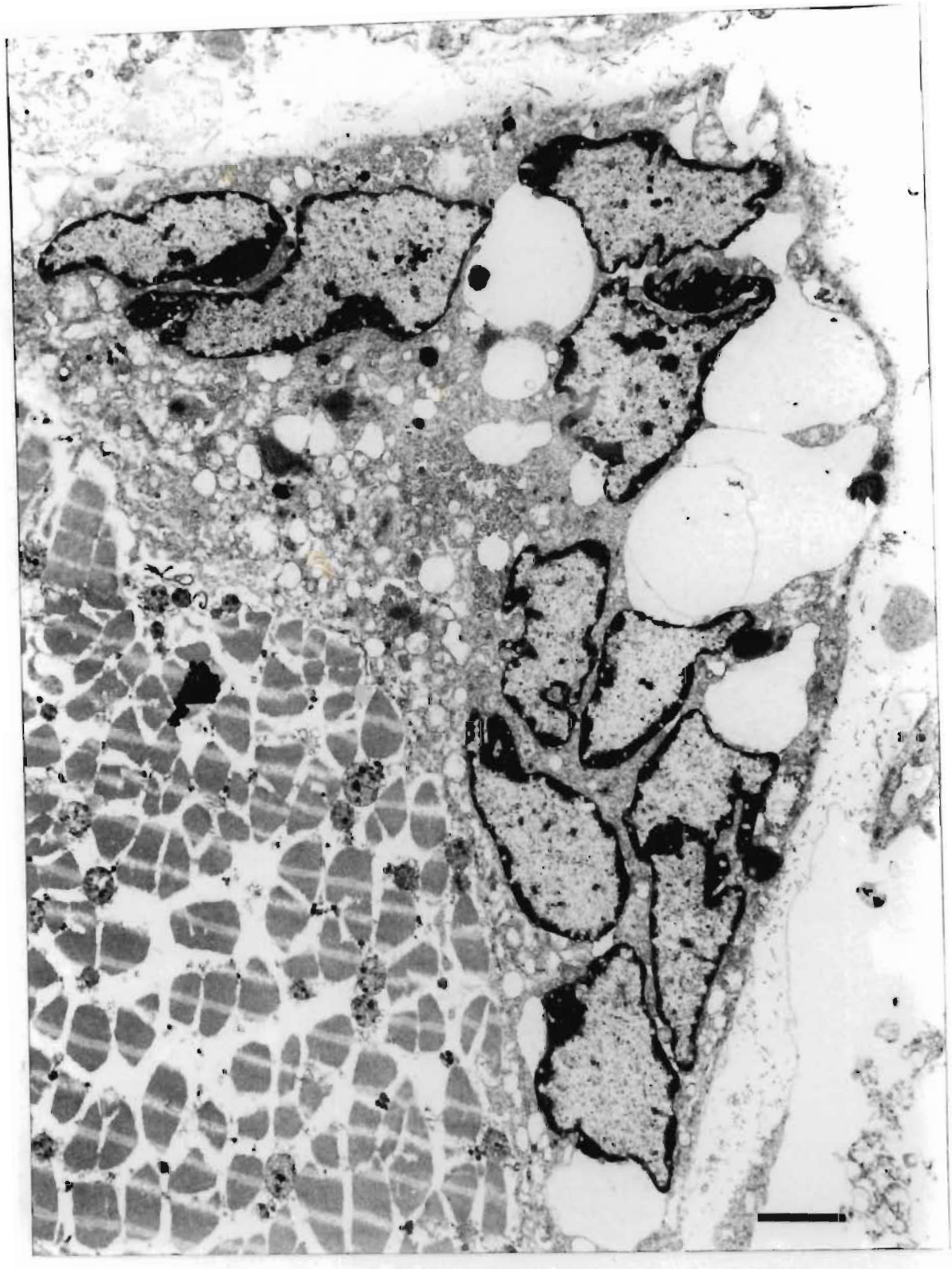


Figure 29. Electron micrograph of hamster explant after 6 days incubation showing a cluster of nuclei in a myotube commonly found at the ends of myofibres. Large membrane bound vacuoles present, perhaps they provide the scaffolding for the nuclear formation. The nuclear morphology is highly irregular and does not suggest mitotic derivation of the nuclei. Bar = 2 μm.

size of the new cytoplasmic bodies formed. The large cytoplasmic bodies apparently gave rise to numerous irregular nuclei (Figs. 26*A*, 26*B* and 27).

The smaller aggregations of sarcoplasmic organelles seemed to be associated with the development of a single nucleus which occupied most of the area of the "new generation cytoplasm" (Figs. 26*C* and 32*B*).

5.5.9.4 "New generation cells"

The "new generation cytoplasm" together with the "new generation nuclei" are suggested as the source of the many cells observed in the later incubation period. The areas previously occupied by mitochondrial aggregations and clusters seemed later to be replaced by cells appearing as the "new generation cells". Some of these cells were often found lying above remains of the mitochondrial clusters (Fig. 28*A*). The terminal parts of myofibres which had greater aggregations of mitochondria and other organelles would appear to be involved with numerous cells (Fig. 28*B*). The cells derived in this way had morphological features in common, and this perhaps depended on their state of physiological maturity. The nucleoplasm was diffuse and granular with nuclear inclusions. The nuclear inclusions are understandable if one takes into account the manner in which the nuclei are derived. The cytoplasm of these cells was dense granular in appearance with very little differentiation. Cytoplasmic inclusions were not uncommon. Their morphology was quite different from the morphology of the sublaminar cells observed during the earlier incubation period.

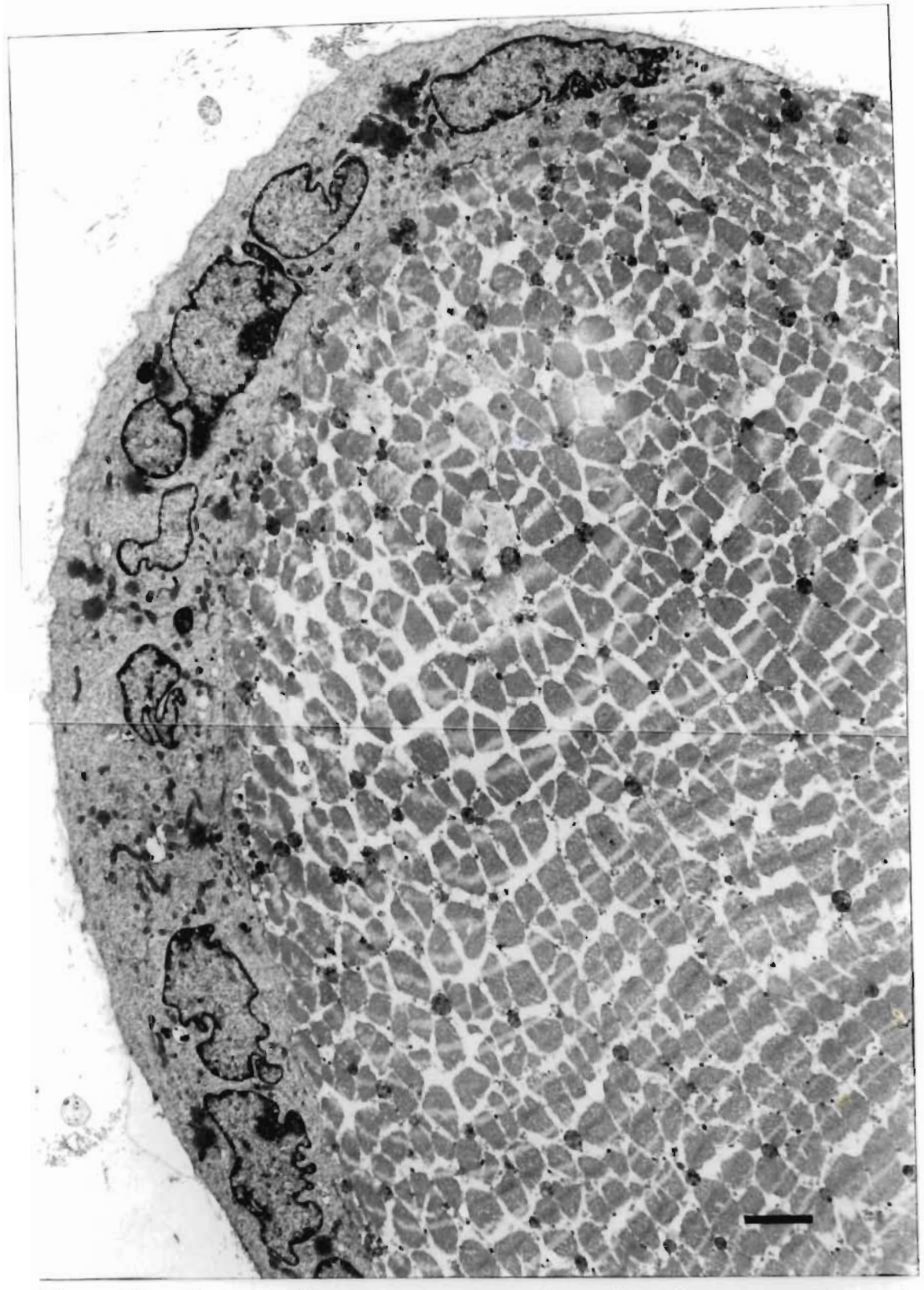


Figure 30. Electron micrograph montage of a multinucleate myotube cap in hamster explant after 6 days incubation. The bizarre shaped multinuclei have what appear to be nuclear buds. The myotube's association with the degenerate myofibre is an attempt at continuous regeneration. Bar = 2 μm.

Some explants of the human muscle exhibited similar derivation of "new generation cells" associated with cytoplasmic organelles (Fig. 32). However the frequency of these new generation structures were not as high as that observed in the hamster explants. Perhaps more would have been seen in the human muscle explants had they been incubated for a longer period. Even the multinucleated forms of irregular nuclei were not observed in the human muscle explants at 10 days of incubation.

Young "new generation cells" with electron dense nuclei and dense granular cytoplasm had similar morphology to those seen in hamster muscle explants. The cytoplasm of the "new generation cell" shown in the human electron micrograph did not contain any visible organelles (Fig. 32*D*). Furthermore, the nucleus of this "new generation cell" contained large electron dense nuclear inclusions which closely resembled residue of organelles.

Close inspection of the nucleus in this cell reveals that the nucleus was not yet completely formed. The nuclear membrane is clearly incomplete and terminates, at the break points, attached to small vesicles. The morphology of this cell alone provides sufficient evidence to dispel doubts that new cells may be generated in association with sarcoplasmic elements of degenerating myofibres by unknown mechanisms.

Schiaffino et al. (1979) observed undifferentiated cells with heterochromatic nuclei deep within the myofibre in rat muscle showing compensatory hypertrophy. Perhaps, these cells observed by them were derived in a similar manner.

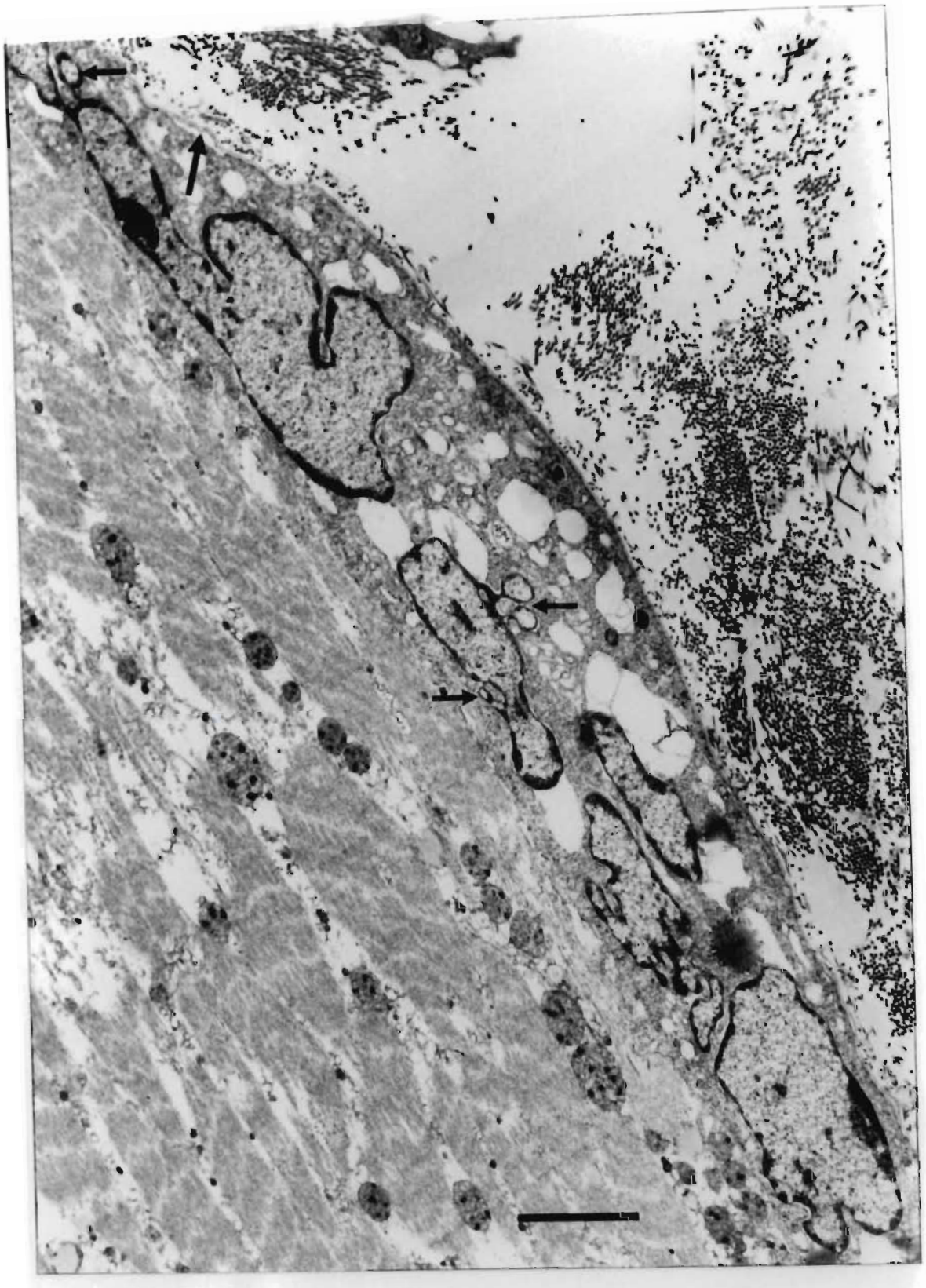


Figure 31. Electron micrograph of hamster explant, after 6 days, showing part of a myotube with bizarre multinuclei below the basement membrane (long arrow) along the lateral surface of the degenerate myofibre. The small arrows point to possible nuclear budding. Bar = 2 μm.

5.5.9.5 Multinucleate myotubes with irregular and bizarre nuclei

During the late incubation period, multinucleate myotubes with an array of irregular and bizarre nuclei were a common feature, being present in the sublaminar position along the length of the myofibre as well as over the terminal parts of the myofibres. The multinucleate myotubes seem to be formed by two mechanisms. The first is that the myotubes at the terminal ends of the myofibres, especially those having a conical appearance, with clusters of irregular nuclei (Fig. 29) and those with chains of nuclei extending along the myofibre (Fig. 31), were perhaps formed from new cytoplasm together with new irregular nuclei which were derived from the clusters of partially formed irregular "new generation nuclei". The cytoplasm of these myotubes had autophagic vacuoles, degenerate mitochondria and large vacuolated areas adjacent to some of the nuclei. These vacuoles appeared to be extended outer nuclear membranes or perhaps empty spaces left by nuclei that may have been lost during the EM processing. The morphological features of the irregular nuclei and the cytoplasm of the myotube closely resembles that of the new cytoplasmic forms with irregular and incomplete nuclei that they were apparently derived from (Figs. 26*B* and 27).

In the second mechanism, multinucleate myotubes with cytoplasm not exhibiting much of the degenerate features described in the first case, were probably formed by the fusion of the mononucleated cells (Figs. 28 and 32*D*) derived from other "new generation nuclei" (Figs. 26*C* and 32*B*). These multinucleate myotubes (Fig. 30) also had similar irregular nuclei and also formed a cap over the terminal part of other myofibres (Fig. 30), and often extended down the length of the myofibres. The nuclear morphology

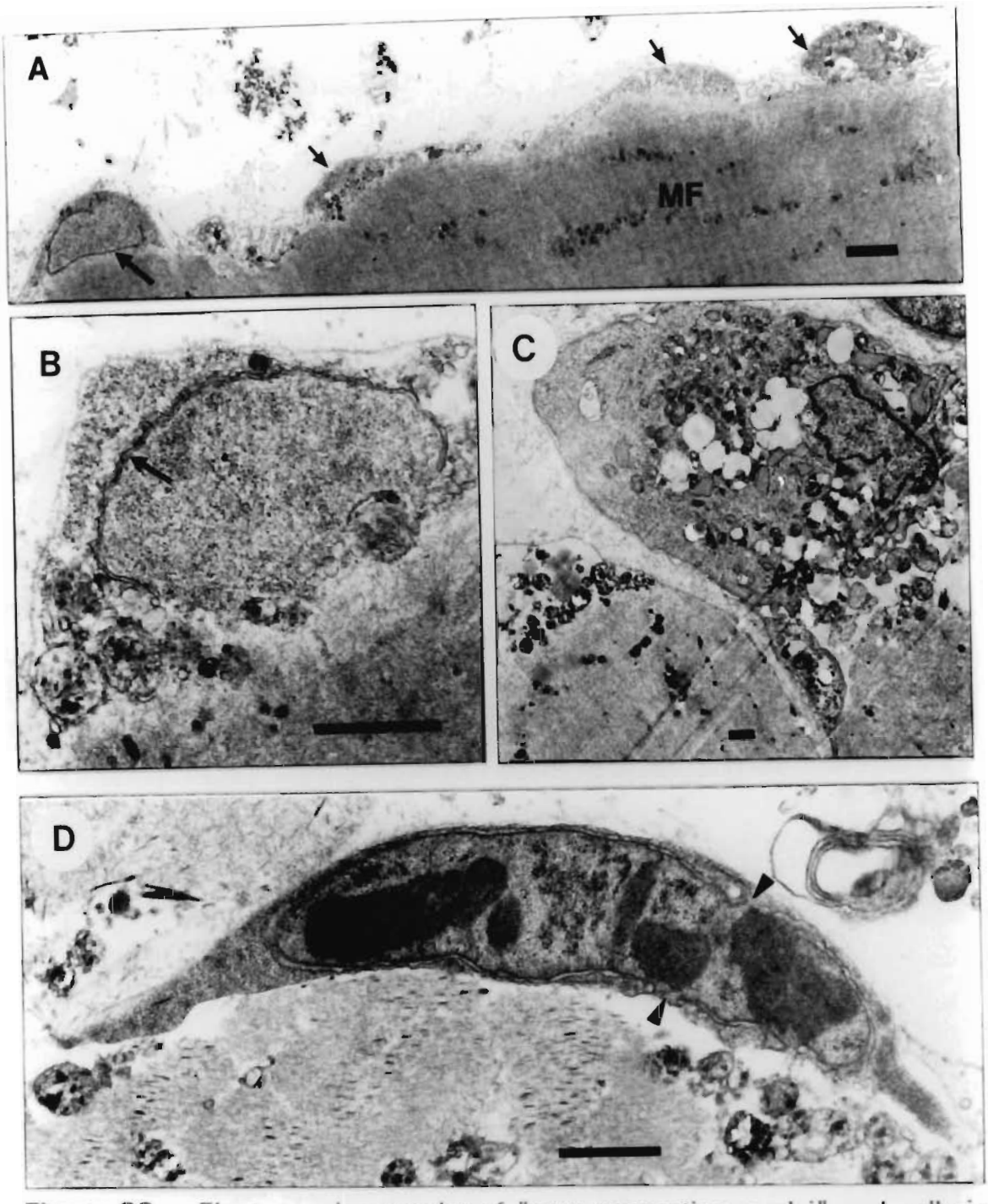


Figure 32. Electron micrographs of "new generation nuclei" and cells in incubated human explants. *A:* New cytoplasmic forms (small arrows), some with a formative nucleus (big arrow) under convolutions of basement membrane of a degenerate myofibre (MF) (4th day). *B:* Another formative nucleus at higher magnification, with clear partially formed double membranes (arrow) (4th day). *C:* Myoblast at the end of a degenerate myofibre, presumed to originate from cytoplasmic organelles of the parent myofibre (9th day). *D:* Young cell with dense granular cytoplasm without any differentiated organelles, within a degenerate myofibre on the 10th day. Nuclear membranes are incomplete at points (arrowheads).

Bar = 1 μm.

of many of these irregular nuclei gave the impression that they were either segmenting or budding of more nuclei (Fig. 31). This again provides evidence for possible amitotic nuclear division occurring in muscle cells. If these multinuclei were formed by conventional mitotic divisions, then an explanation would be required for their irregular and bizarre outlines.

Although these sublaminar myotubes had so many irregular nuclei, the myofibrillar content of these myotubes were scanty when compared to the amount of myofibrillar material found in multinucleate myotubes formed during the initial phase of regeneration where myotubes were formed by fusion of "regular" myoblasts. Perhaps the incubation time was insufficient to have observed more developing myofibrils seeing that many of these multinucleate myotubes were formed in the latter part of the incubation period.

Lash et al. (1957) described similar irregular shaped and tightly packed nuclei in muscle regenerating after injury. They did not see any mitotic figures in the regenerating muscle, nor did they find any evidence of how the irregular nuclei originated. Allbrook (1962) also reported seeing these irregular multinuclei and suggested that they were due to infoldings of the nuclear membranes. Similar bizarre forms of nuclei were reported for mitochondrial myopathy with clinical characteristics of progressive muscular dystrophy in a Japanese female infant, and in the same study numerous, large and bizarre mitochondria were observed (Nagaura et al., 1990). These irregular myonuclei appearing in pathological tissue was evidence that "new generation nuclei and new generation cells" were also possible in the, **in vivo**, situation.

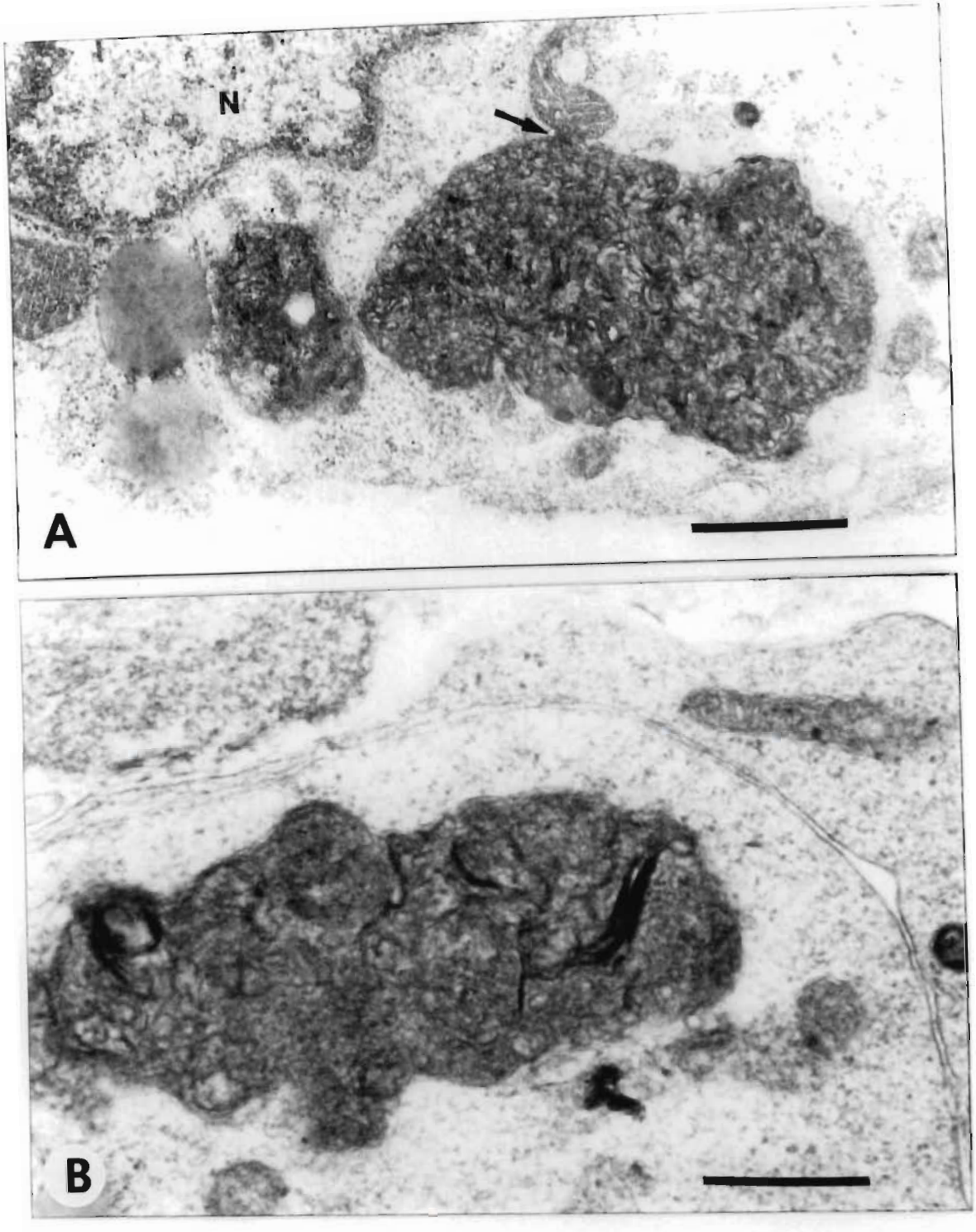


Figure 33. Electron micrographs of hamster explants after 7 days incubation showing 'giant' mitochondria in *A* & *B*. These mitochondrial bodies, in the cytoplasm of presumptive myoblasts, appear to be the fused product of a number of mitochondria. Fusion between mitochondria (arrow) is revealed in *A*. Bar = 0,5 μm.

Perhaps when normal myoblast resources are depleted in pathological tissue, other mechanisms, as described above, are put into action in an attempt to complement the dwindling myofibre population of diseased muscle.

5.5.10 Giant mitochondria

Giant mitochondrial bodies were observed in the cytoplasm of some of the myoblasts (Fig. 33). These giant mitochondria were apparently the product of fusion of smaller mitochondria, and this is said because morphological representations of apparent fusion between mitochondria were observed (Fig. 33*A*). The crystal membrane structures, even though they were disorganised, were clearly visible in the giant mitochondrial bodies. There was no sequential evidence observed to suggest any possible function of these bodies.

Apparent fusion of mitochondria were commonly observed in the sarcoplasmic compartment of the regenerating muscle in the current study. Fusion of mitochondria was also demonstrated in the flight muscle of the blow fly by Gregory et al. (1968) who considered insects suitable for muscle development studies because the sequence of development and senescence in insects was completed in a matter of days.

5.5.11 Some morphological features in regenerating cells similar to those described in pathological muscle

Some of the myotubes growing in a cone formation at the distal ends of myofibres (Fig. 34*A*) exhibited what appeared to be "generation workshops". A number of areas in the electron micrographs showing the

Figure 34. Electron micrographs of 'generation workshops' in a young multinucleate hamster myotube on 7 days incubation. *A:* Montage of a conical multinucleate myotube at the end of a degenerate myofibre. Some structural cytoplasmic features of the myotube show in *B* dense cytoplasmic bodies (1), membrane forms partially around electron dense chromatin like material (2), myelin figures (3) and dense elongated mitochondria. These are shown in more detail in Fig. 23. Bar = 2 μm in *A* and 1 μm in *B*.

possible derivation of structural elements, identified in pathological muscle, is revealed in the electron micrographs presented (Figs. 34 and 35).

Fibrous myelin like structures associated with electron dense cytoplasmic or lysosomal-like body and another neighbouring structure, wrapped around by apparent formative membranes containing mitochondrial and lysosomal bodies associated with vacuoles filled with glycogen perhaps signalling the beginnings of a new nucleus (Fig. 35*A*), were apparent regenerative features and not degenerative features.

Generation of new nuclei, shown by partial membrane formations around electron dense chromatin-like material, by mechanisms already discussed, were also apparent in the cytoplasm of the conical myotube (Fig. 35*B*). It appeared as though the generation of new nuclei was being continued in this myotube which appeared to be a "new generation cell".

Mitochondrial remnants, in which the crystal formation was apparent, seemed to be associated with myelin-like or fibrous structures observed in pathological muscle (Fig. 35*C* and 35*D*). Many of these structures, observed in dystrophic tissue, were evidence that the muscle cells were attempting to regenerate and therefore it would be erroneous to regard them as features of degeneration.

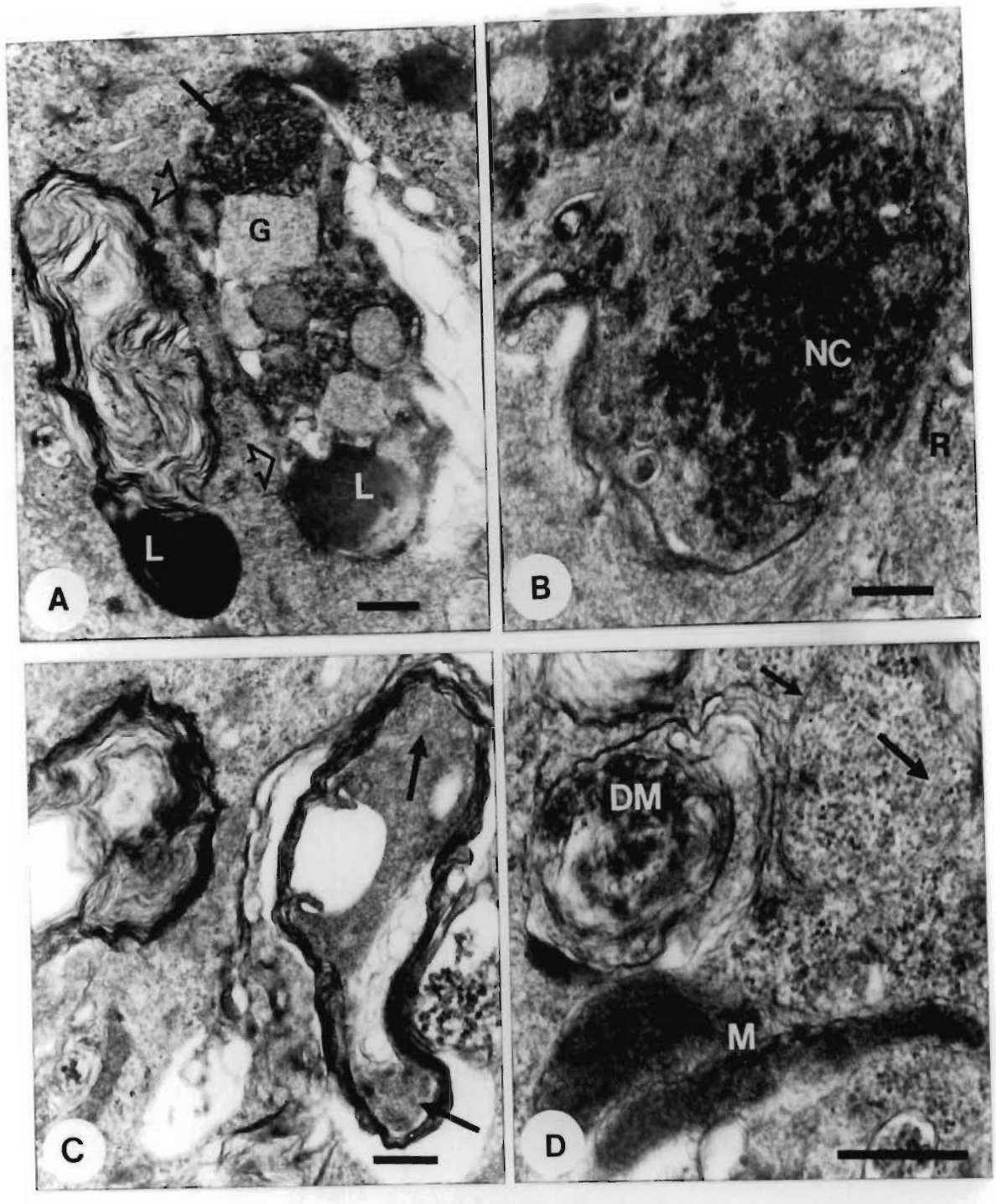


Figure 35. Magnification of some of the 'generation workshops' observed in areas 1 to 4 of Fig. 22. *A:* area 1 - a dense lysosomal like body (L) adjacent to a fibrous structure. Near this, is another structure (open arrows) containing a dense mitochondrion (arrow) and vacuoles with glycogen (G). There is also a lysomal body much like a degenerated mitochondrion. This structure, wrapped around by formative membranes, perhaps signals the beginning of a new nucleus as in *B*, area 2 - where membranes surround nuclear chromatin like material (NC). The myelin like figures in *C*, area 3 - are associated with mitochondrial remnants (arrow). *D:* area 4 - Origin of filaments from a degenerate looking mitochondrion (DM). Dense elongated bodies (M) appear to be new mitochondrial formations. short arrow, microtubules; long arrow, microfilaments. Bar = 0,5 μm.

5.6 GENERAL COMMENT

Konigsberg (1963) expressed caution in the analysis of results from culture which might be completely an atypical response unrelated to the processes occurring ***in vivo***, and that the progeny of differentiated tissue may undergo modulation in response to exposure to the artificial environment. One has to be mindful also of the absence of various growth factors, such as fibroblast growth factor (DiMario and Strohman, 1988; DiMario et al., 1989; Smith and Schofield, 1994), nerve growth factor (Oh and Markelonis, 1979; Baron et al., 1994), platelet-derived growth factor (Tidball et al., 1992) and other obvious humoral factors such as hormones, in the culture environment. Furthermore, the development of secondary myotubes (young myofibres) from primary myotubes were shown to be dependant on neurally evoked electrical stimulus (Ashby et al., 1993) which was absent in the culture environment of the current study. This perhaps was one of the reasons why the multinucleate myotubes, formed during the earlier incubation period from conventional myoblasts in the muscle explants, did not achieve full maturity to myofibres.

Nevertheless, since the time of caution expressed in 1963 by Konigsberg, the explant techniques were used successfully to grow primary cells and promote fusion of myoblasts in cultures leading to the development of multinucleated myotubes. These myotubes continued development leading to young striated muscle fibres which were observed to undergo contraction in cultures, thus proving the efficacy of the culture techniques to promote differentiation of muscle cells, ***in vitro,*** (Tautu and Jasmin, 1982; Delaporte et al., 1984; Jasmin et al., 1984) although they did not go to full maturity with a full complement of myofibrils. Therefore, if primary cells from the

explants can undergo fusion and differentiation in culture conditions, it was expected that conditions within the explants, although not ideal, were suitable to demonstrate the early regenerative changes. Nerves were not considered as a requirement for the early differentiation and morphogenesis, but only for the functional differentiation and maintenance of cells in the muscle (Carlson, 1979).

However, the human muscle specimens which served as the normal control samples, were placed into fixative for EM study, immediately after being removed from the patients. Therefore, the human muscle control specimens were not subjected to any of the deficiencies such as growth factors or artificial environmental influences of the laboratories. Many of the transformational changes of the myonuclei leading to the development of primordial myoblasts were observed in these uncultured human muscle. Therefore, technically speaking, the derivation of myoblasts from myonuclei being observed in the ***in vivo*** situation leaves little room for doubt.

In the final analysis, the main difficulty of any of the techniques tracing the regenerative changes, is presented by the inability to perform a sequential study of the same myofibres. Although the interpretations of the morphological changes observed in the electron micrographs during muscle regeneration appeared obvious or logically conclusive, the findings need to be validated by ongoing research in the future by histochemical or immunocytochemical methods. Confirmation of the "new generation cell" is eagerly anticipated from other research centres once these findings are published. Furthermore, the genetic composition of these cells need to be established by those qualified to do so. To embark on new work in search of solutions for the above, the local laboratory facilities will have to be updated and finance will have to be raised to meet this requirement.

CHAPTER 6

CONCLUSION

An intense EM study of the regenerative changes occurring in cultured and uncultured hamster and human muscle explants lead to the following conclusions:

a. During muscle regeneration, euchromatic myonuclei become electron dense and granular or heterochromatic.

b. The morphological change of the myonuclei heralds the transformation of these myonuclei to the precursor cells of muscle regeneration or future myoblasts. As the number of myoblasts increased with incubation time the number of myonuclei declined.

c. In the initial stages of development, many of the myoblast were still partially attached to the parent sarcoplasm. In their later stages there was complete membrane separation between the myoblast and the parent sarcoplasm. Therefore, many of these cells may be found located between the basement membrane and the plasma membrane. This is how the classical satellite cell is formed and obtains its location below the basement membrane in adult muscle. The cytoplasm around the young cells are apparently new cytoplasm which perhaps is secreted by the transforming myonuclei.

d. During regeneration, the myoblasts increase in number, undergo fusion and form multinucleate myotubes.

e. It is not certain at this stage how the myoblasts proliferate as mitotic figures were not observed. However, there was visible evidence suggesting that myonuclei may be undergoing amitotic division by segmentation of the nucleus. This would explain many of the beaded nuclei, observed in muscle, having a prominent nucleolus in each segment. If myonuclei were to increase in number by amitotic division, then the proliferation of the myoblasts could be explained.

f. Myotubes may also be formed from mononucleated myoblasts. The nuclei of many myoblasts provide morphological evidence of apparent amitotic division. This answers the intriguing question on the issue of how myonucleation occurs in myotubes.

g. Under culture conditions, the myotubes, although they formed young myofibrils, failed to achieve full maturity to myofibres. Midway through their development, they exhibited degenerative features.

h. Myoblasts also behave as phagocytes. Phagocytic-appearing cells were shown to have distinct developing myofibrils in their cytoplasm denoting their myogenic status. The phagocytic behaviour was attributed to the cells apparently reutilising sarcoplasmic elements as, perhaps, nutrients rather than performing the function as scavengers like the macrophages. The phagocytic myoblastic character was just another stage in the development of myoblasts.

i. From morphological features observed in the cultured muscle it appears as though, that when the myoblast resources from myonuclear derivation are depleted, the muscle has the potential to develop "new generation cytoplasm and new generation nuclei" both of which lead to the formation of "new generation cells" from yet unknown mechanisms.

j. Many of the sublaminal myotubes containing clusters or chains of irregular and bizarre nuclei are formed by fusion of mononucleated newly generated cells or from newly generated multinuclei within newly generated cytoplasm. This is perhaps the way in which bizarre nuclei were derived in pathological muscle.

k. The irregular or bizarre myonuclei thus formed were capable of amitotic division either by nuclear segmentation or budding.

l. Many of the structures such as nuclear inclusions, the electron dense cytoplasmic bodies, myelin figures and Z line streaming were seen in the regenerating muscle. Their presence in pathological tissue therefore signified that regeneration was in progress in that muscle.

m. There was evidence of the association of myelin figures with mitochondria.

It is worth remembering, that in embryological development, the skeletal muscle precursor cells or myoblasts initially occurred singly. These myoblasts proliferated mitotically and finally fused with each other giving

rise to mature multinucleate muscle fibres (Fischman 1972). It was therefore not unreasonable to accept that the skeletal muscle had the potential to undergo a somewhat reverse process in times of stress, whereby the myonuclei became activated, gathered cytoplasm and developed into new myoblasts which again multiplied, fused and formed new multinucleate muscle fibres, if and when required. The myonuclei of mature muscle must not be regarded as inert structures, but as vital instruments for dynamic homeostatic control of the myofibre. The mitochondria also seem to play a role more than we understand at the present time.

Although satellite cells have not been irrefutably proven to be the precursor cells of muscle regeneration, all current research, especially the myoblast transplant therapy, engaged in therapeutic trials in search of a cure for muscular dystrophy, focus on the satellite cells. Researchers involved in the above trials assume that satellite cells with the normal genetic complement will migrate into the recipient's myofibres and fuse with them to form a new population of cells to overcome the genetic defect. The capability of satellite cells to migrate between myofibres is contradicted in many of the research papers (Bischoff, 1979; Shultz et al., 1985; Satoh et al., 1993). Those who believed in satellite cell migration did so because they could not explain the large number of cells at sites of injury by satellite cell proliferation alone. These researchers did not consider the possibility of new cells being derived from myonuclei. However, poor migration of injected donor myoblasts was said to be the cause of poor positive dystrophin response in myofibres of recipients undergoing myoblast transplant trials (Satoh et al., 1993). The poor preliminary results on myoblast transplant therapy can be attributed to this wrong focus on satellite cells. If the

satellite cells are not the precursor cells of muscle regeneration, as is shown by the current study, then researchers working on future therapies for muscular dystrophy need to rethink their strategy and focus on the myonuclei.

Finally, it is hoped that the information gained in this study, documenting for the first time the early regenerative events in cultured human and hamster muscle explants with the methods described, will help towards a better electron microscopic interpretation of the morphological changes observed in the muscle of experimental subjects undergoing therapeutic trials, as well as in pathological muscle from patients. Furthermore, if the results of this study on regeneration are confirmed in the future at other research stations, the researchers involved in the search for a cure will have to re-orientate their therapeutic design and targets. It is hoped that the results of the current study will assist in the future management and treatment possibilities of muscular dystrophy.

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Errata

Page 154, line 13: Myonuclei in control specimens appeared as though they were imbibing myofibrillar elements (Fig. 7*C*).

Page 156, line 16: Figures 8*B* and 8*C* seem to show imbibition of cytoplasmic material.

Page 180, line 3: Imbibition of cytoplasmic elements seemed to occur in Figures 8*B* and 8*C*.

Page 232, line 14: Mastaglia and Walton (1971) also suggested that regenerating cells may "*actually reutilise breakdown products of necrotic sarcoplasm*".

Page 263, line 10: "by mechanisms already discussed" to be replaced with "as discussed in 5.5.9.3"

APPENDIX I

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EM Investigation of Myoblast Origin in Regenerating Hamster Skeletal Muscle Explants

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This study attempted to dispel the confusion that exists in the understanding of the origin of myoblasts during muscle regeneration. Regenerating hamster muscle explants from cultures were studied under the EM on 4 consecutive days, after incubation. Preincubation specimens served as controls. Revelations were that euchromatic myonuclei underwent dense granulation and activation after incubation. Presumptive myoblasts (PM) lying clearly within the myofibre increased in numbers with incubation time. Some myonuclei showed partial transformation towards a PM. This study concluded that myonuclei transformed into myoblasts during the process of muscle regeneration and that the PM, produced from a myonucleus, was a stage in the development of the satellite cell (SC) in regenerating muscle. These SC, myoblasts from myonuclear origin, proliferated, fused, and formed multinucleate myotubes that matured into myofibres which replaced damaged muscle. Findings of this study may have new implications for the proposed myoblast transplant or gene transfer therapy, both of which, whilst being possible answers for muscular dystrophy, depend on a sound knowledge of muscle regeneration mechanisms.

INTRODUCTION

Muscular dystrophy, an insidious muscle wasting disease, to the present day defies modern medical science. Myoblast transfer/muscle cell transplant (Partridge *et al.*, 1989, 1991; Brooke, 1990; Karpati, 1990; Law *et al.*, 1990) and genetic engineering (Acsadi *et al.*, 1991) are currently viewed as possible future treatments.

Keeping in mind the objectives of these therapies which depend heavily on the regenerative ability of skeletal muscle, this project, using hamster muscle explants in culture, was undertaken to investigate the origins of myoblasts during muscle regeneration. The need for this work was clearly shown by Sloper and Partridge (1980) who concluded: "The source of the mononuclear muscle cell precursors is still uncertain. The idea that an undifferentiated, as it were embryonic, satellite precursor cell can persist through adult life, lying between plasma and basement membranes of the muscle fibre, has gained wide acceptance; but it has not entirely superseded the view that myoblasts can arise by segregation of differentiated myonuclei. It remains possible too that local connective tissue cells and, again, circulating cells may have an accessory role in myogenesis."

MATERIALS AND METHODS

Reagents. The following reagents were used: (1) Dulbecco's modification of Eagle's medium without glutamine with 4.5 g dextrose/liter (DMEM) (Flow Laboratories). (2) Chick embryo extract (CEE)—50% in Earle's balanced salt solution without phenol red (Flow Laboratories). (3) Fetal bovine serum (FBS) (virus and mycoplasma tested—Whittaker M A Bioproducts). (4) Antibiotic–antimycotic mixture (100×) (AA) (GIBCO Laboratories). (5) L-Glutamine (200 m*M*) (GIBCO Laboratories). (6) Gelatin (BDH Chemicals). (7) Hanks' balanced salt solution without calcium and magnesium (HBSS). (8) Alcohol (70%) made with sterile deionised double-distilled water. (9) Sterile deionised double-distilled water. (10) Complete nutrient medium (CNM) was made up of 100 ml DMEM, 15 ml FBS, 5 ml CEE, 2 ml L-glutamine, and 1 ml AA. (11) Standard reagents for preparation of specimens for electron microscopy—glutaraldehyde, cacodylate buffer, osmium tetroxide, alcohol, lead citrate and uranyl acetate and araldite.

Animals. Five Normal Syrian hamsters were used (see Table 1). The guidelines for animal care and experimentation, as laid down by the Medical Research Council of South Africa, were observed.

Culture procedure. Strict aseptic techniques were adhered to. Dissections and culture preparations were performed in a culture cabinet (Laminaire Bio-Hazard 4BH, Bino Instrumentation). Each hamster was rapidly decapitated with a guillotine (Ealing, U.S.A.). Each decapitated hamster was immersed in a beaker of 70% alcohol to ensure surface sterilisation before dissection.

Approximately 500 mg of thigh muscle was surgically removed and placed in cold HBSS containing 2% AA in a sterile petri dish. The muscle was sectioned into smaller pieces with a sterile blade and all visible fat and connective tissue were teased out with a pair of sterile needles. The muscle fragments were washed twice in HBSS + AA which was then replaced by CNM. The muscle was then minced with a blade into approximately 1 mm^3 pieces and washed twice again in CNM to remove remaining blood cells and debris. Approximately 150–200 mg of muscle pieces or explants were then transferred with a large bore pipette into each of two culture flasks (25 cm^3), which were pretreated with 0.5%

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TABLE 1
Hamster Data and Myonucleus/Satellite Counts in C

H no	Wt/g	Sex	Myonucleus %	Satellites %
1	156	F	95	5
2	72	M	92	8
3	85	M	92	8
4	130	M	96	4
5	75	M	90	10

Note. C, control.

gelatin. Explants were fed with 8 ml CNM warmed to 37°C. Gentle agitation of flasks ensured even dispersion of the explants before they were incubated (Laminaire incubator, Bino Instrumentation) in air containing 5% CO2 and 80% humidity at 37°C.

Hamster skeletal muscle explants were maintained in culture under this controlled laboratory environment (Jasmin *et al.*, 1984) for a period of 10 days. Each culture flask was gently agitated once a day to prevent adhesion of the explants to the gelatinised surface of the flask. Old CNM was changed for new after the fifth day. Cultures were inspected daily using an inverted microscope (ELIZA Tokyo).

Electron microscopy. A few muscle explants with apparent intact structure were removed from the cultures, every day from Day 1 after incubation, for electron microscopy. Preincubation muscle specimens served as controls. The specimens were fixed using 2.5% glutaraldehyde in nutrient medium (pH 7.4) over 1 hr at room temperature. Nutrient medium was used to minimize shock and retraction of fibres. The specimens were then subjected to the standard procedure of washing with buffer, postfixing with 1% aqueous osmium teroxide, washing with water, dehydration with alcohol, and embedding in araldite. Four resin blocks were prepared from each hamster specimen every day. Semi-thin sections for light microscopy and ultrathin sections for EM were prepared using glass knives on a ultramicrotome (Reichert Ultracut). Semi-thin sections stained with toluidine blue were viewed using a research light microscope (Nikon Optiphot) with a camera attachment for photomicrographs. Sections on grids were stained with uranyl acetate and lead citrate. A grid prepared from each block was studied using a Zeiss EM10B electron microscope.

RESULTS

Light Microscopy

Cultures. Initially the explants were semi-transparent, but they began to become opaque from the second day of incubation. The myofibres in some of the explants were convoluted, due to retraction. Myogenic cells or presumptive myoblasts (PM) migrating or sprouting from the muscle explants were evident 3 to 4 days after incubation. Many of these migrating cells adhered to the gelatinised flask, became spindle shaped, and formed typical myoblast colonies (Yasin *et al.*, 1977). Some cells were pleomorphic with irregular cytoplasmic outlines, and these most probably represented fibroblasts.

Semi-thin sections. Uninucleate cell forms increased markedly after the first day of incubation. It was difficult to precisely distinguish between myonuclei and early uninucleate cells at the light microscope level because the young uninucleate cells with scanty basophilic cytoplasm appeared morphologically similar to myonuclei. It was also difficult to deduce the characteristics of the intensely staining or seemingly pyknotic nuclei.

Multicells in chains or multinucleate cells developing along the myofibre appeared from the third day after incubation. Again, it was not possible to differentiate whether each nucleus was present in independent cells or if they belonged to the same syncytium.

Electron Microscopy

Controls. Ultrastructure of preincubation specimens which served as controls appeared normal with myofibre structural integrity remaining intact, keeping in mind the mechanical injury incurred during preparation of the explants. Satellite cell counts, obtained by the number of cells below the basement membrane per 100 myofibre nuclei counted, ranged between 4 and 10% with the average being 7% (Table 1). The SC counts compared favorable with other work (Allbrook *et al.*, 1971; Snow, 1979; Wakayama and Schotland, 1979; Ishimoto *et al.*, 1983) using different experimental animals. The true SC described by Mauro (1961) must lie between the basement membrane and the sarcolemma of the myofibre and be an independent cell.

Most of the myonuclei were typically euchromatic (Fig. 1A) as found in normal myofibres (Dubowitz, 1985), but there were also a few myonuclei (Figs. 1B and 1C) with dense peripheral heterochromatin resembling the nucleus of SC denoting activity.

Day 1 after incubation. Most of the myonuclei (Fig. 1E) appeared dense and granular with a homogeneous distribution of nuclear material. Often these electron-dense nuclei were seen in the company of many mitochondria indicating perhaps their energy requirement. Very few typical euchromatic myonuclei were observed. Typical SC were rare. More PM with scanty cytoplasm (Figs. 2C and 2D) were present within the myofibre below the basement membrane. Vesicles to help form future membranes of the myoblast to separate it from the parent myofibre (Hay, 1959) were often seen (Fig. 2C). The scanty cytoplasm in Fig. 2D reveals an early stage of development of a PM from a myonucleus. The presence of myofibrils between the basement membrane and this early myoblast indicates its location within the myofibre.

Day 2 after incubation. No typical euchromatic myonuclei were observed. Some myonuclei displayed invaginations of the nuclear membrane (Figs. 1D and 1E). Electron-dense material was concentrated in the nucleoplasm proximal to the invaginations of some of the nuclei (Fig. 1D). All of the other myonuclei observed were dense and granular. The number of PM found lying within myofibres increased markedly.

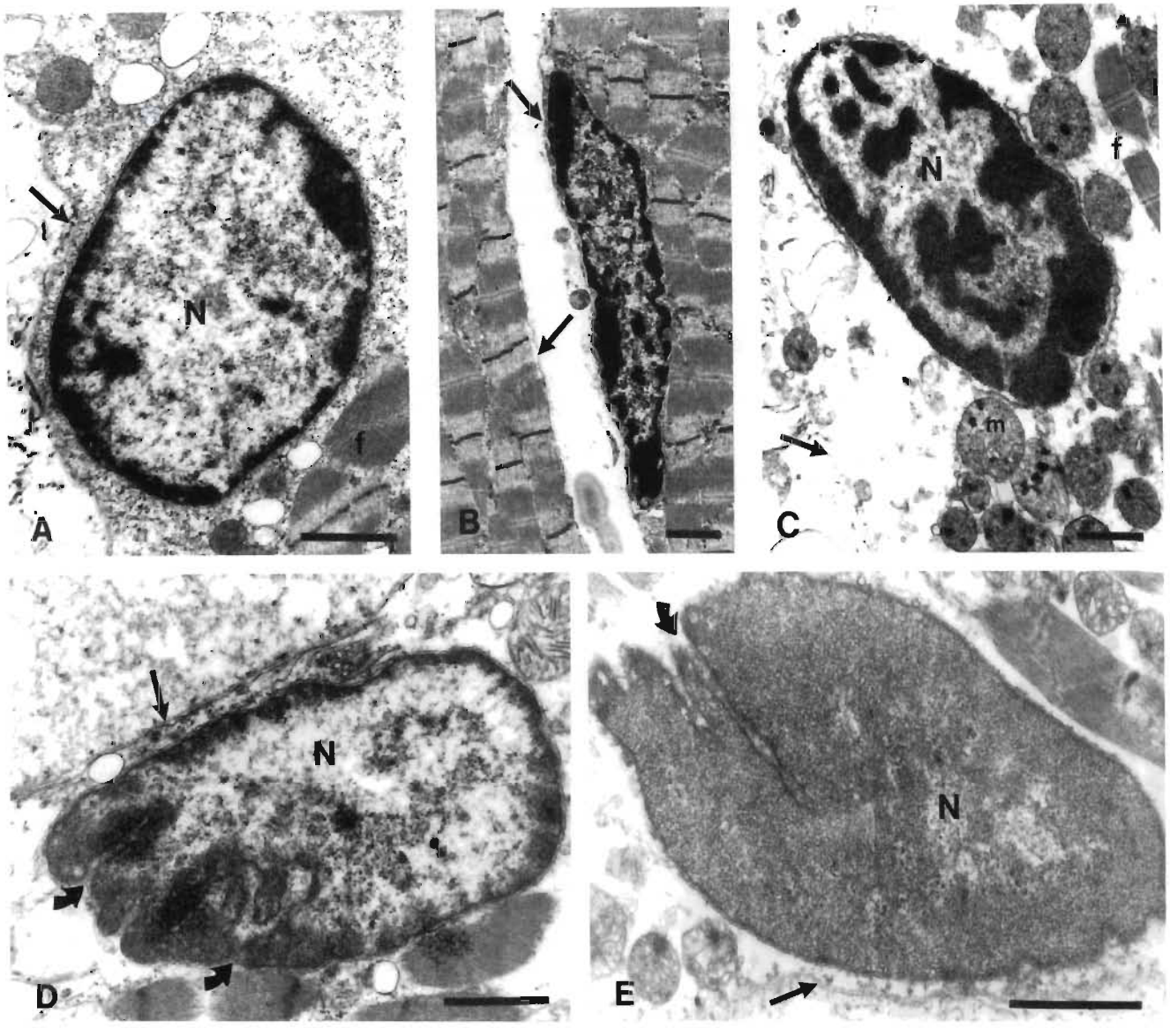

FIG. 1. Electron micrographs (A–E) represent some of the myonuclear morphology observed in explants during 4 days of incubation. Typical euchromatic myonucleus (A) was present mainly in the control. Myonuclei (B, C) with dense peripheral heterochromatin, resembling the satellite cell nucleus, were observed in controls and 1 day after incubation. (D and E) After incubation, invaginations are displayed (thick arrows). (D) This micrograph has electron-dense material in regions of the invaginations and is probably a stage in the transformation to (E) with dense homogeneous granular material. N, myonucleus; m, mitochondria; f, myofibrils; thin arrows, basement membrane. Bar = 1 μm.

Day 3 after incubation. Myonuclei which were few in number were similar to those in Figs. 1C–1E. Peripherally located uninucleate PM with scanty cytoplasm, distinctly lying within the parent myofibre, were still present in the explants (Fig. 3A). Cytoplasmic elements of the parent myofibre were clearly observed between the basement membrane and the myoblast cytoplasmic membrane (Fig. 3A and 3B), lending evidence that the myoblasts must be within the myofibre syncytium. The poor organelle development in these myoblasts with scanty cytoplasm reflects their immaturity.

The observation of remnants of sarcolemma in some of the parent myofibre between the basement membrane and the myoblast cytoplasmic membrane (Fig. 3B) provides the evidence that the myoblast must have its origin from a myonucleus within the myofibre. Only parts of the sarcolemma of the parent myofibre were observed because of the obvious disruptive and degenerative changes the myofibre is subjected to during culture.

Myoblasts in chains and at different stages of development were present below the basement membrane along the length of many myofibres. Different stages of myoblast development were indicated by varying densities of cytoplasm and organelle development. Cells in clusters were found at the distal ends of some fibres forming a cap (Fig. 4A). Binucle-

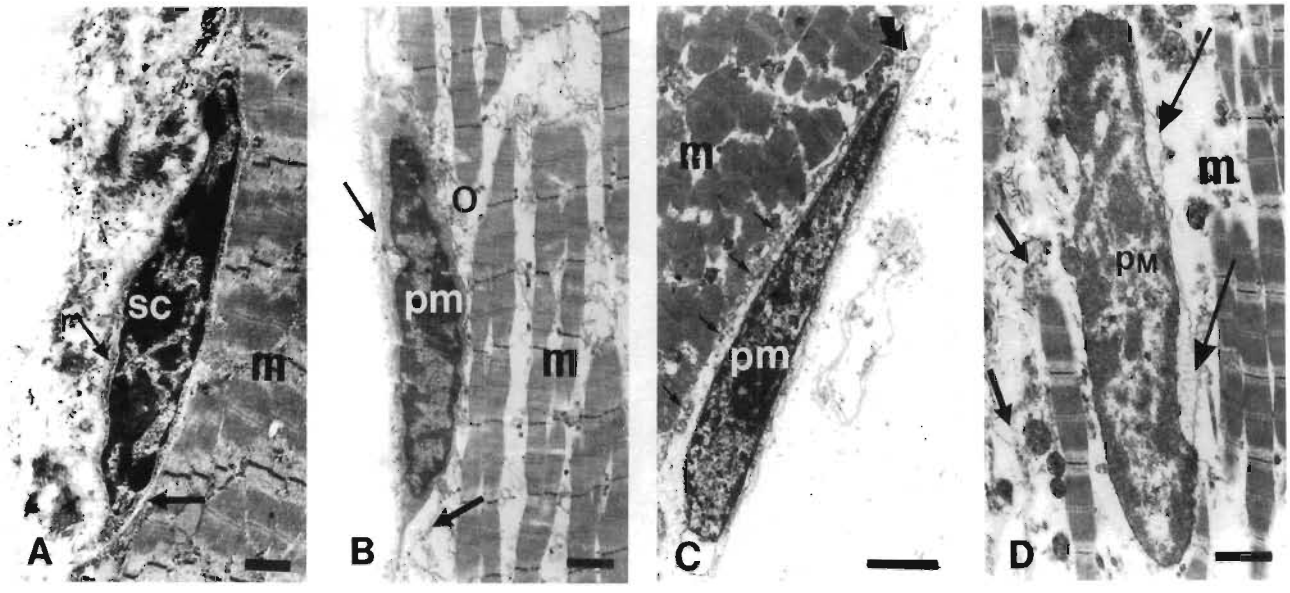

FIG. 2. Electron micrographs. (A) A typical satellite cell (sc), having a nucleus with dense heterochromatin and scanty cytoplasm, lying between the basement membrane (thin arrow) and sarcolemma (thick arrow) of the myofibre (m), observed in controls. The presumptive myoblast (pm) in (B), observed in controls and after incubation, resembles a satellite and lies below the basement membrane (thin arrow) but is within the myofibre. Clear separation is visible at one end between the sarcolemma (thick arrow) of the myofibre and the cytoplasmic membrane of the presumptive myoblast, but no distinct separation is seen in the region of o. Presumptive myoblasts (C, D) were observed after incubation. Cytoplasm (thick arrow) in (C) is visible only at one end of the nucleus, indicating the early transformation of the myonucleus to a myoblast. Note the presence of vesicles (thin arrows) between the myofibrils of the myofibre and the presumptive myoblast with no distinct separation between the two in this region. (D) A presumptive myoblast in an early stage of development having scanty cytoplasm (long arrows). Note the presence of myofibrils between the basement membrane (short arrows) and the presumptive myoblast, indicating that the presumptive myoblast is within the myofibre. Bar = 1 μm.

ate and multinucleate myotubes (Fig. 4B) also appeared in some explants. Mitochondrial aggregations (Fig. 4A) clearly point to the regenerative activity within the myofibres.

Day 4 after incubation. Day 4 was similar to Day 3, except that regenerative cells increased. Early myofibrils were observed in the cytoplasm of some myotubes (Fig. 4B). Z lines were identified in some of the myofibrils which usually began to develop in the peripheral region of the myotubes.

EM observations and discussion of the regeneration process in the explants from the fifth day after incubation will be presented in a future communication.

DISCUSSION

Muscle explants in culture afford an excellent opportunity to study *early regenerative changes,* on account of the easy accessibility and availability of the explants for study on a day to day basis. Such study, otherwise, is difficult in an intact animal that would have to be subjected to repeated surgical procedures that would be painful, both for the animals and the researcher. Also the muscle explants were free of other "outside-muscle" influences (Askanas, 1979), being grown in a controlled laboratory environment. The problems of blood clots and phagocytes encountered as a result of experimental injury caused in the animals, in previous *in vivo* experiments on regeneration (Allbrook, 1962; Baker and Poindextor, 1991), were absent, making the early tracing of events involved in regeneration more meaningful. The disadvantage encountered in the use of explants was that after incubation they were extremely delicate and fragile and therefore easily susceptible to disruption of the structural integrity if not handled with care.

It has now come to be accepted by many (Church, 1969; Mastaglia and Kakulas, 1969; Moss and Leblond, 1971; Schmalbruch, 1976; Bischoff, 1979; Nichols and Shafiq, 1979; Ontell, 1979; Snow, 1979; Allbrook, 1981; Schultz *et al.,* 1985; Carpenter, 1990) that satellite cells, embryonic remnants of myogenesis, were reserve, undifferentiated cells, capable of differentiating into myoblasts promoting muscle regeneration in mature muscle.

There are others (Lee, 1965; Reznik, 1969; Hess and Rosner, 1970; Teravainen, 1970; Walker, 1972) who believed that myonuclei had the potential to be activated in times of muscle stress, to surround themselves with cytoplasm, and finally to segregate as a new cell from the parent myofibre by a process called dedifferentiation (Hay, 1959).

An intense electron microscope investigation of the muscle explants in the present study helped to clarify and identify the ultrastructural changes

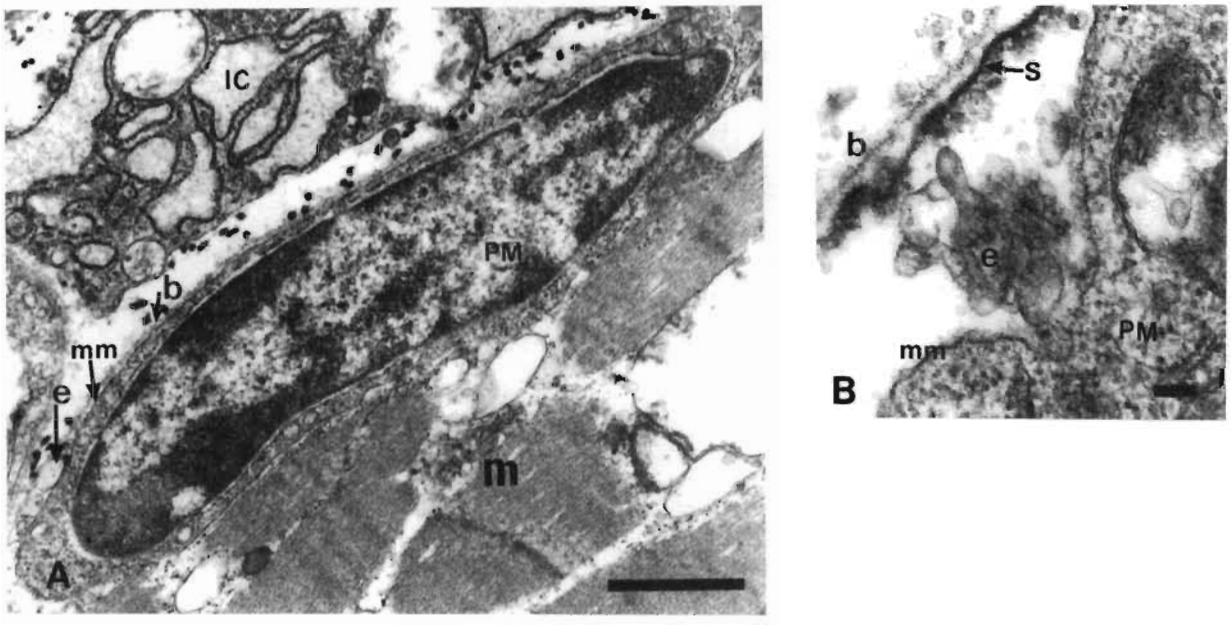

FIG. 3. Electron micrographs. (A) Presumptive myoblasts (pm) distinctly shown below the basement membrane (b) and as part of the myofibres (m). Cytoplasmic elements (e) of the myofibre lying between the basement and the myoblast cytoplasmic membrane (mm) lend further evidence that the myoblast must be within the myofibre syncytium. These presumptive myoblasts observed after incubation, have little cytoplasm without complex organelle development. (B) Remnants of sarcolemma (s) in some of the parent myofibres were observed clearly at places between the basement and the myoblast cytoplasmic membrane, indicating that the presumptive myoblast must have its origin from a myonucleus within the myofibre. IC, interstitial cell. Bar = 1 μm in (A) and 0.1 μm in (B).

which occurred early in the regenerative process. The ultrastructural integrity of the muscle fibres in the control explants appeared normal taking into consideration the mechanical injury sustained by the explants during their preparation for culture. As incubation proceeded, myofibres in the explants began to exhibit oedema, fibrillar disorganisation, mitochondrial degeneration, and regeneration, all of these changes being reported in other work concerning muscle regeneration (Allbrook, 1962; Reznik, 1969; Lipton, 1979; Baker and Poindextor, 1991). In the midst of these seemingly degenerate myofibres, the regenerative processes were set in motion.

The present study focused attention on satellite cells, myonuclear changes, and especially the origin of myoblasts in muscle explants maintained in culture up to 4 days.

Apart from the typical euchromatic myonuclei, there were myonuclei indicating transformation, with dense peripheral chromatin which was typical

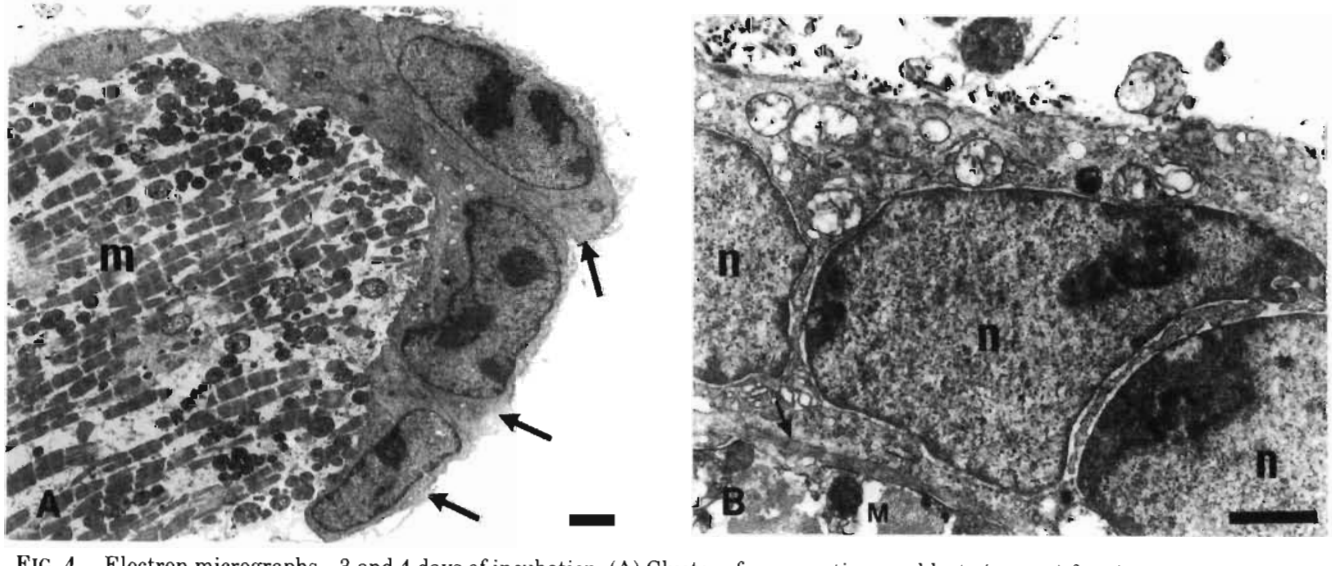

FIG. 4. Electron micrographs—3 and 4 days of incubation. (A) Cluster of presumptive myoblasts (arrows) forming a cap at the end of a myofibre (m). (B) Multinucleate myotube lying beneath the basement within the myofibre. Developing myofibrils (arrow) are present. n, nucleus. Bar = 2 μm in (A) and 1 μm in (B).

of nuclei of SC. When seen in longitudinal sections of intact myofibres, these myonuclei could easily be mistaken for SC. Dense granular myonuclei, some of them with invaginations of their membranes, were a common feature only in the incubated explants. These dense myonuclei were also observed in other regeneration studies (Lash *et al.,* 1957; Allbrook, 1962), and these were possibly identified as pyknotic nuclei (Witkowski, 1977; Snow, 1979).

Invaginations of membranes also occurred in some euchromatic myonuclei with electron-dense material in the nucleoplasm adjacent to the walls of the invaginations. These myonuclei seemed as though they were imbibing cytoplasmic elements in the area of the invaginated troughs. This would explain the electron-dense material in the region of the invaginations, and would perhaps also explain the origin of many dense granular myonuclei. The active nature of these myonuclei may also explain the presence of the many mitochondria found close to them, probably there to meet the energy requirement.

Previous work (Lash *et al.,* 1957; Witkowski, 1977) suggested that the early stages of muscle regeneration were denoted by myonuclear activity. Therefore, the dense myonuclei observed by them and in this study were probably euchromatic myonuclei that were transformed to a stage preceding the formation of PM of myonuclear origin.

It is agreed that the SC in mature muscle served the function of precursors of myoblasts, but there was no conclusive evidence that these cells were embryological remnants of myogenesis, lying in reserve. On the contrary, the present study supports the view that there was a development of new SC from myonuclei whenever the need arose for regeneration. There must be continuous, slow turnover of these cells in normal muscle, which is why they were always reported to be present (Allbrook *et al.,* 1971; Wakayama and Schotland, 1979; Watkins and Cullen, 1988), but in very small numbers, counts varying according to age and the physical condition of the muscle.

Some PM (Fig. 2B) closely resembled SC, the only difference being that these PM were within the myofibre. Morphological similarity between these two cells lent support for the view that the PM must be a stage in the development of SC. After the separation of the PM from the parent myofibre, by the formation of new cytoplasmic membranes between them, the PM will then be a new SC ready to promote regeneration. This view was strongly supported by Hess and Rosner (1970).

A number of PM also had nuclear morphology with variable scattered chromatin perhaps denoting transitional stages between PM with dense granular nucleus and the SC with nucleus having dense peripheral heterochromatin.

The strongest evidence for the derivation of a PM from a myonucleus of the myofibre comes from the observation of remnants myofibre sarcolemma between the basement membrane and the presumptive myoblast, and also from the location of myofibre cytoplasmic elements between the basement membrane and the cytoplasmic membrane of the PM.

The supposition, by many researchers, that SC gave rise to the myoblasts that were found fused with the subjacent myofibre was true for the process of myonucleation of muscle fibres in the normal course of myogenesis in the young. That concept, applied here in regeneration of mature muscle, would imply that these myoblasts from SC origin were trying to resuscitate the degenerate fibre. This was unlikely, with the overwhelming evidence presented in regeneration studies which indicated that restoration of damaged muscle was by the way of developing new myotubes.

The SC of mature muscle, therefore, was a myoblast which was previously a PM. Possible mechanisms of cleavage or segregation for myoblast development from myonuclei were already described (Lee, 1965; Hess and Rosner, 1970; Reznik, 1976). But, the ultrastructural characteristics of the scanty cytoplasm of the PM, with little or no organelle development, as observed in this study suggested that the cytoplasm may in fact be secreted by the active myonucleus in the initial stages of development of the myoblast. Light micrographs revealed uninucleated cells in regenerating myofibre to have intensely basophilic cytoplasm which was distinctly different from the subjacent cytoplasm of the myofibre. If myoblasts were formed by the cleavage mechanism (Hay, 1959; Hess and Rosner, 1970; Reznik, 1976) where the myonucleus with some cytoplasm separates from the myofibre by membrane formation between them, then the cytoplasms of both the new myoblast and the parent myofibre should have similar staining and structural features. This needed further research.

In vivo regeneration studies (Allbrook, 1962; Reznik, 1969; Hess and Rosner, 1970) on rabbits, mice, and guinea pigs strongly supported the findings of the present study. Allbrook (1962) described "a type of cell commonly found in the position of a subsarcolemmal nucleus in a muscle fibre." He went on to say that this cell had a dense granular nucleus and it was "otherwise similar to a subsarcolemmal muscle nucleus." It was unfortunate that EM study was reported only after 90 hr of injury to the muscle. It was likely that these subsarcolemmal cells would have been observed sooner had Allbrook resorted to electron microscopy earlier. Reznik (1969) also showed similar cells after 68 hr. In that study, it was stated that the majority of myonuclei in the degenerative phase of the fibres became pyknotic and disappeared. These pyknotic nuclei might be the dense

granular nuclei referred to in this study, and perhaps their disappearance could be explained by the fact that they transformed into cells.

The main body of support for satellite cells being myogenic precursors came from [^{3}H]thymidine studies (Moss and Leblond, 1971; Snow, 1979). Snow (1979) shows a pyknotic nucleus (Fig. 7 p. 97) which was labelled with [^{3}H]thymidine and for want of better interpretation, the nucleus was regarded as part of a degenerate satellite. This labelled nucleus was most likely an activated dense granular myonucleus on its way to becoming a myoblast, and the many large mitochondria in close association with it denoted its energy requirement for such a transformation. Moss and Leblond (1971) also demonstrated labelled myonuclei, but only after 24 hr onwards and concluded that these myonuclei must have come from SC which were incorporated into the myofibre. It seemed remote that these labelled satellite cells would have lost their cytoplasm in such short time to assume the identity of true myonuclei. The more plausible explanation might be that these labelled myonuclei were the activated dense granular myonuclei which underwent transformation. The transformation process must incur an increase in the DNA content in these dense nuclei and therefore their positive labelling.

On the other hand, Walker (1972) using the same isotope labelling concluded that myonuclei were responsible for the development of myotubes during muscle regeneration. Schultz *et al.* (1985) suggested that the large increase in SC at the site of injury was due, in part, to migration of satellites from uninjured areas. They interpreted their result on the assumption that all labelled nuclei they counted must belong to SC, which of course is not necessarily true.

The chains of myoblasts under the basement membrane and the myoblast clusters at ends of some myofibres, which at this stage reveal definite organelles, undergo fusion to form multinucleate myotubes with a progressively increasing quantity of early myofibrils some of which revealed Z line development. At this stage in the study, how proliferation of myoblasts took place cannot be accounted for as no mitotic changes were observed in the many myofibre-associated cells or nuclei in this study.

CONCLUSION

It is worth remembering, that in embryological development, the skeletal muscle precursor cells or myoblasts initially occurred singly. These myoblasts proliferated mitotically and finally fused with each other giving rise to mature multinucleate muscle fibres (Fischman, 1972). It was therefore not unreasonable that the skeletal muscle had the potential to undergo a somewhat reverse process in times of stress, whereby the myonuclei became activated, gathered cytoplasm, and developed into new myoblasts which again multiplied, fused, and formed new multinucleate muscle fibres, if and when required. Such is the conclusion of the present study. The myonuclei of mature muscle must not be regarded as inert structures, but as vital instruments for dynamic homeostatic control of the myofibre.

It is hoped that the information gained on the origin of myoblasts during muscle regeneration in explants will help towards the future management and treatment possibilities of muscular dystrophy, where myoblast transplant or gene transfer methods are contemplated.

An investigation of the myoblast origin in regenerating human skeletal muscle explants in culture, using the same methods described for the present study on hamster skeletal muscle, is presently in progress in our laboratory.

Special thanks to Mrs. T. Naicker and Mrs. S. Bux of the EM unit of the Faculty of Medicine, University of Natal, for their technological expertise and kind cooperation. Thanks, also, to Professor G. Jasmin and Dr. C. Tautu of the Department of Pathology, University of Montreal, and to Dr. R. Yasin of The Institute of Neurology and Neurochemistry, University of London, for personal tuition in muscle culture techniques. This study was supported in part by grants from the Medical Research Council of South Africa and Natal University Muscular Dystrophy Research Fund.

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APPENDIX II

EM EVIDENCE OF MYOBLAST ORIGIN IN REGENERATING HUMAN SKELETAL MUSCLE EXPLANTS

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ABSTRACT

EM study of cultured human skeletal muscle explants on 10 consecutive days after incubation made possible a record for the first time, the early events occurring during regeneration. After incubation, normal myonuclei underwent activation and dense granulation. Some myonuclei showed early transformation to presumptive myoblasts. The conclusion was that myonuclei transformed into myoblasts which developed into satellite cells (SC). These SC of myonuclear origin, proliferated, and fused forming myotubes that matured into myofibres, replacing damaged muscle. The findings have new implications for the current myoblast / cell transplant and gene transfer therapy research which may provide possible answers for muscular dystrophy in the future.

INTRODUCTION

Medical science continues its battle against muscular dystrophy, a debilitating disease. Myoblast transplant (Partridge *et al.*, 1989; Karpati, 1990; Law *et al.*, 1990; Gussoni *et al.*, 1992; Huard *et al.*, 1992) and gene transfer (Acsadi *et al.*, 1991) methods are currently being investigated as possible future therapies. Because these therapies depend on muscle regeneration, this study attempted to clarify the position of the precursor cells or presumptive myoblasts (PM) during regeneration in human skeletal muscle
and to ascertain if the findings are similar to that found in the earlier work on hamster muscle (Naidoo, 1992). The need for this work was clearly shown by Sloper and Partridge (1980) who concluded: "The source of the mononuclear muscle cell precursors is still uncertain."

MATERIALS AND METHODS

Gluteal, tibialis, flexor digitorum, sternohyoid and rectus muscle samples, from five different subjects of both sexes ranging between the ages 23 and 65 years were used in the study. Even though the ideal would have been to obtain muscle from normal subjects, muscle samples were obtained from patients undergoing surgery for conditions other than muscle diseases. Because these muscle samples came from sites of injury, the control specimens, uncultured showed some signs of early regeneration.

Human muscle was subjected to the same techniques described previously (Naidoo, 1992), except that incubation was prolonged for 10 days as human muscle in *vitro* studies take longer to show regenerative changes. In brief, the culture technique involved the mincing of cleaned muscle and growing them in flasks containing DMEM base medium with chicken embryo extract, foetal bovine serum and antibiotic / antimycotic mixture. Incubation was at 37^0C in humid air containing 5% CO_2. Four muscle explants (ME) from each culture, removed on 10 consecutive days after incubation, were prepared for EM study. Preincubation ME served as controls. Ethical clearance was obtained for this work.

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RESULTS AND DISCUSSION

Light microscopy of cultures revealed that the explants under incubation behaved similar to hamster muscle (Naidoo, 1992), except that the human muscle seemed more susceptible to

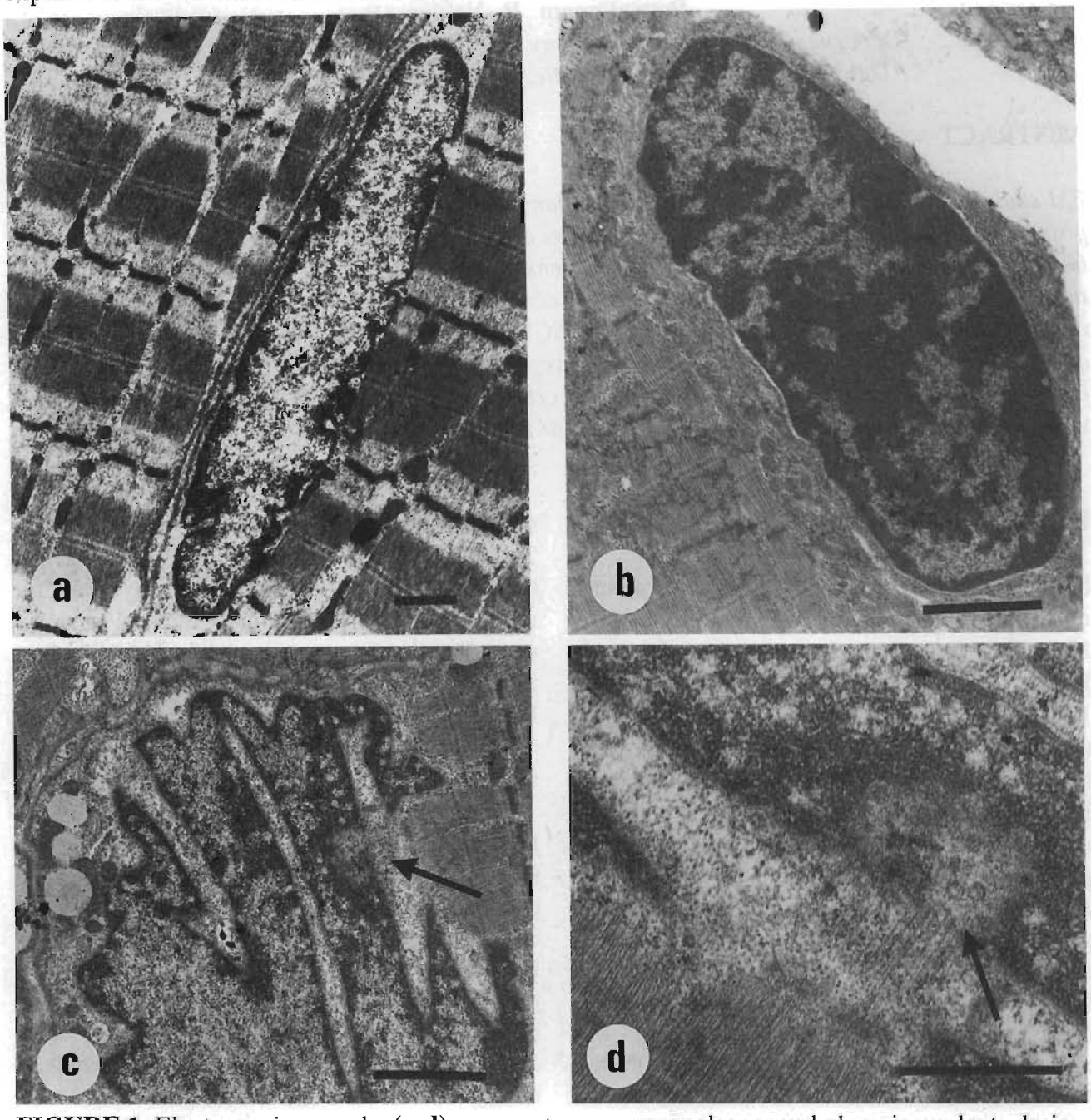

FIGURE 1. Electron micrographs (**a-d**) represent some myonuclear morphology in explants during culture. Typical euchromatic myonucleus (**a**) was present in controls. Myonuclei (**b**) with dense heterochromatin, resembling satellite cell nucleus were observed in controls and for 4 days after incubation. Invaginated nucleus (**c**), showing a magnified part of the same in (**d**), was a common feature after incubation. They seemed to imbibe cytoplasmic elements (arrow) and this explains the electron dense material in regions of the invaginations. This nucleus is probably a stage in the transformation to (**b**) with electron dense material. Bar = 1 *u*m in (**a-c**) and 0.5 *u*m in (**d**).

retraction in the first few days of culture and stabilising thereafter. Myogenic cells or PM sprouting from the ME were evident between 6 and 7 days after incubation. Identification of nuclear and cell forms was clear only with the EM.

Typical euchromatic myonuclei (Dubowitz, 1985) were present in myofibres of controls (Fig.1a). Myonuclei (Fig. 1b) indicating transformation, with dense peripheral and scattered central heterochromatin were seen in some controls, but mainly in myofibres during the first few days after incubation. Dense heterochromatic myonuclei present in controls, indicating signs of regeneration, may be attributed to muscle samples being obtained from sites of injury and the muscles exposure to drugs. When seen in longitudinal sections, these myonuclei could easily be mistaken for SC.

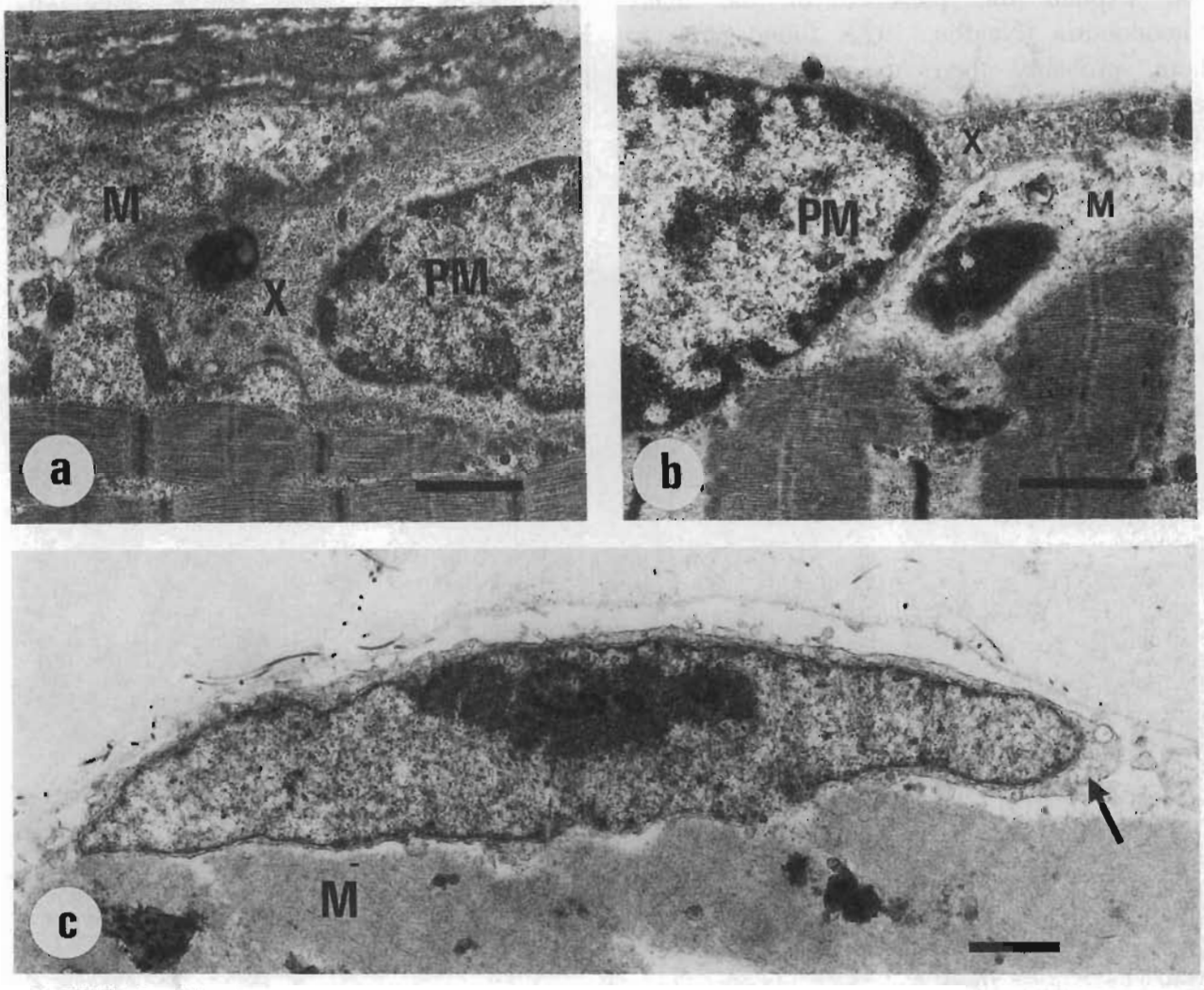

FIGURE 2 . Electron micrographs (**a-c**) represent presumptive myoblasts, **PM** in different stages of development. (**a**) Reveals early development of dense cytoplasm **X** around part of the nucleus with no distinct membrane separating it from cytoplasm, **M** of the parent myofibre. (**b**) also shows, at one end of the nucleus, new cytoplasm **X**, with different density from the cytoplasm of the parent myofibre, **M.** The markings of early cytoplasmic membranes beginning to separate the PM from parent myofibre are present only between the zones of cytoplasm. (**c**) A full nucleus of a PM with little cytoplasm (arrow), lying within the parent myofibre, M. In all the above PM, the cytoplasm is scanty and its immaturity is denoted by little or no organelle development. Bar = 1 μm.

Myonuclei (Fig. 1c) with deep invaginations, which seemed to imbibe cytoplasmic elements in the area of invaginations (Fig. 1d) were a surprisingly common feature of regenerating muscle, as reported in earlier work (Naidoo, 1992). This imbibition would explain the electron dense material adjacent to invaginations, and would perhaps also explain the origin of the many electron dense or heterochromatic myonuclei. The active nature of these myonuclei also explain the presence of the many mitochondria (Naidoo, 1992) found close to them, probably there to meet the energy requirement. Previous research (Lash *et al.*, 1957; Witkowski, 1977) suggested that the early stages of muscle regeneration were denoted by myonuclear activity.

As incubation progressed, the numbers of myonuclei declined whilst the number of cell forms (Fig. 2c) increased. Earlier research on animals (Reznik, 1969) reported the disappearance of myonuclei due to degeneration. The present study attributes this disappearance of myonuclei to their transformation to cells. Partial transformation of myonuclei to (PM), (Fig. 2a-b) were seen for 6 days after incubation. The PM were positioned within the parent myofibre with no complete and continuous membrane separating them from the parent myofibre. Early markings of the cytoplasmic membrane (Fig. 2b) beginning to

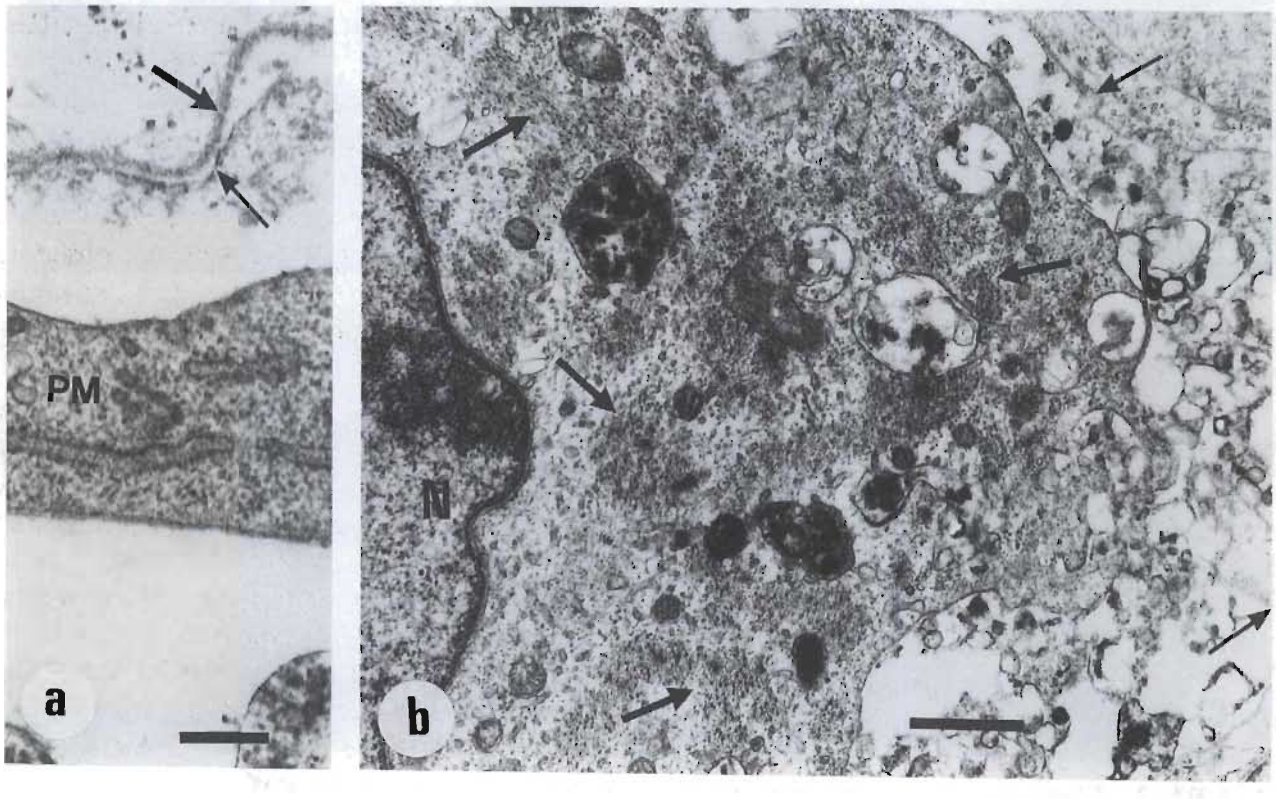

FIGURE 3. (**a**) Shows part of cytoplasm of a PM with well developed organelles. The presence of remnants of the sarcolemma (thin arrow) of the parent myofibre between the basement membrane (thick arrow) and the PM indicates clearly that the PM has its origin from within the myofibre. (**b**) is a cross section of part of a myotube, with the cytoplasm around the nucleus N showing distinct developing myofibrils (thick arrows). Basement membrane, (thin arrows). Bar = 0.25 *u*m in (**a**) and 0.5 *u*m in (**b**).

initiate separation were often observed only in the regions where new cytoplasm of the PM was observed. Morphological similarity between some PM (Fig. 2c) and the typical SC lent support for the view (Naidoo, 1992) that the PM must be a stage in the development of SC. Some PM, having nuclei with scattered dense chromatin perhaps denote transitional stages between PM with dense granular nucleus and the SC with nucleus having dense peripheral heterochromatin (Dubowitz 1985).

The observation of remnants of myofibre sarcolemma (Fig. 3a) between the basement membrane and the presumptive myoblast provides stronger evidence for the derivation of PM from a myonucleus. Only parts of the sarcolemma of the parent myofibre were observed because of the obvious disruptive and degenerative changes the myofibre is subjected to during culture.

Ultrastructural characteristics of the scanty cytoplasm of the PM, with little or no organelle development suggested that the cytoplasm may in fact be secreted by the active myonucleus in the initial stages of development of the myoblast and not by cleavage mechanisms (Lee, 1965; Hess and Rosner, 1970; Reznik, 1976) or by dedifferentiation (Hay, 1959). Light micrographs of regenerating myofibres revealed uninucleated cells with intensely basophilic cytoplasm, distinctly different from the subjacent cytoplasm of the myofibre. If myoblasts formed by cleavage mechanisms, then the cytoplasms of the myoblast and the parent myofibre should have similar staining and structural features.

Between 8 and 10 days after incubation myoblasts in chains (Naidoo, 1992), at different stages with varying densities of cytoplasm and organelle development appeared below the basement membrane along the length of many myofibres. Binucleate and multinucleate myotubes (Naidoo, 1992) with distinct myofibrils (Fig. 3b) also appeared at this time. Mitochondrial aggregations (Naidoo, 1992) observed in the explants after 6 days of incubation clearly pointed to regenerative activity within the myofibres.

The current consensus (Sloper and Partridge, 1980) is that satellite cells (SC), considered remnants of embryogenesis (Mauro, 1960), lying between the basement membrane and the myofibre sarcolemma are reserve myoblasts which persist through life. During muscle stress these SC proliferate and fuse forming myotubes which mature into myofibres replacing damaged muscle.

Many researchers assumed that SC gave rise to the myoblasts found fused with the subjacent myofibre. That assumption applied here in regenerating muscle explants, implied that these myoblasts from SC origin, were trying to resuscitate the degenerate myofibres. This was unlikely, as evidence in regeneration studies indicated that restoration of damaged muscle was due to development of new myotubes. Also, the PM occurred singly and were not observed in pairs in the early stages to indicate derivation by mitotic division of the SC.

Main support for SC, embryonic remnants, being myogenic precursors came from [^{3}H]thymidine studies (Moss and Leblond, 1971; Snow, 1979). These researchers also showed nuclei, within the myofibre, which were labelled with [^{3}H]thymidine. For want of better interpretation, the labelled nucleus was regarded by Snow as part of a degenerate satellite and by Moss and Leblond as a nucleus of a SC already incorporated into the myofibre. These labelled nuclei were most likely activated dense granular myonuclei undergoing transformation.

Few researchers (Reznik, 1969; Hess and Rosner, 1970; Walker, 1972) believed myonuclei also had the potential to transform into myoblasts to assist in regeneration. Walker (1972) also using the same [^{3}H]thymidine

labelling contradicted the findings of the other labelling studies in concluding that myonuclei were responsible for myoblasts in regeneration. The new method (Naidoo, 1992) of EM study using human muscle explants in culture from the time of incubation revealed that myonuclei do have the potential to transform into myoblasts.

Earlier research techniques, involving the mincing and smashing of muscle and leaving them as implants within the animals used, resulted in blood clots, poor circulation, necrotic debris and migration of phagocytes at the site of injury. This made difficult early EM study (Allbrook, 1962; Baker and Poindextor, 1991) which was done only after 4 days. The use of cultured muscle explants overcame the problem of blood clots, poor perfusion, necrotic debris and migrating phagocytes or "outside-muscle" influences (Askanas, 1979) thus making possible EM investigation of the early processes of regeneration from the 1st day after incubation. Mendell *et al.* (1972) attempted an EM study on cultured human muscle, but did not observe the transformation of the myonuclei perhaps due to the now outdated method of culture technique used by them.

On account of the easy accessibility and availability, muscle explants in culture afford an excellent opportunity to study early regenerative changes on a day to day basis. Such study, otherwise, is difficult in an intact animal that would have to be subjected to repeated surgical procedures. The disadvantage encountered in the use of explants was that after incubation they were extremely delicate and fragile, and therefore easily susceptible to disruption of the structural integrity if not handled with care.

The results of this study propose that myonuclei of mature human skeletal muscle have the potential to transform to myoblasts during the process of muscle regeneration. This evidence of the origin of myoblasts during muscle regeneration will assist the current research on the treatment possibilities of muscular dystrophy where myoblast transplant or gene transfer therapies are contemplated.

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APPENDIX III

would bring was not reflected by a corresponding commitment from all the parties involved.

Third, the husbandry of riverine rabbits in captivity poses several problems. This is an easily stressed species, of which individuals are easily injured while being handled. They have shown an abnormally high mortality rate and several of the captive individuals are not breeding successfully. Preliminary observations on the sperm of captive males suggest that at least some of them are not highly fertile. Such an unplanned, uncoordinated breeding programme with an animal species subject to serious breeding limitations is doomed to failure.

The wild riverine rabbit population is so small that it is unlikely to survive without human intervention. However, despite these initial setbacks, the number of remaining wild rabbits, and the available technology and financial resources are more than sufficient for implementing a well-planned, scientifically based breeding programme, provided that the following conditions are observed.

1) A single institution needs to take full responsibility for the implementation of a properly planned breeding programme. The De Wildt Centre is ideally placed to take this responsibility. The management of this facility is aided by a private advisory council, including prominent individuals in academia and conservation.

2) It is vital to have a scientific understanding of the husbandry and other problems related to breeding these rabbits. Studies on the reproduction and social behaviour of the animals in captivity would greatly contribute to the success of the breeding programme.

3) Wild-captured founders for the breeding programme need to be used. Dippenaar and Ferguson[4] suggest that a founder stock of 16 rabbits be used, in order to ensure sufficient genetic variation.

4) It is essential to attempt an experimental release of a few individuals into proven habitats for the rabbits. As captive rabbits are fed on pellets and lucerne and live in unnatural conditions, it is important to know whether these animals can be successfully reintroduced into the wild. Intensive long-term monitoring of the released rabbits would be needed. As the Karoo National Park does not contain habitats in which riverine rabbits have been encountered in the past, certain farms in the vicinity of Victoria West seem to be more suitable sites for such an experimental release. In addition, this would have the beneficial effect of involving local farmers in the exercise.

5) Given the slow rate of increase experienced with captive animals, the possibility of using embryo transplantation as a means of the rapid breeding of these animals needs to be investigated. The extensive knowledge base applicable to domestic rabbits in this regard should prove to be relevant.

Three factors are vital for the conservation of these animals: scientific research, management in captivity as well as in the wild, and extension to the owners of private farms. It would be deplorable if the riverine rabbit became extinct because of our inability to plan and execute an integrated conservation programme involving captive breeding and release. It would also do little for the credibility of those academics who pay so much lip service to developing a theory of conservation biology but ignore the practicalities.

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Correspondence

Macrophagic morphology of myoblasts in hamster skeletal muscle explants

Sir,— I have investigated the macrophagic or myogenic status of fusiform cells found below the basement membrane in regenerating myofibres, using cultured muscle explants (ME). This electron microscope study revealed that many of the sublaminar cells, whilst morphologically appearing as macrophages, were possibly myogenic cells or myoblasts. Their myogenic status was indicated by the presence of morphological structures characteristic of developing myofibrils in their cytoplasm.

Many early *in vivo* regeneration studies on skeletal muscle of animals[1–3] regarded the fusiform cells, occupying a position below the basement membrane of the muscle fibres, to be myogenic or satellite cells.[4] Other research[5,6] suggested that many of these fusiform cells may be macrophages or phagocytes. Yet other work[7,8] indicated that myogenic cells did have the capacity to behave like phagocytes. My study used regenerating skeletal muscle explants from culture to clarify the nature of these fusiform cells.

Six healthy hamsters (*Mesocricetus auratus*) ranging between 72 and 156 g in mass, were used. In brief, approximately 200 mg of ME, obtained from the thigh muscle of each decapitated hamster, was prepared for culture. The culture medium was made up of Dulbecco's modification of Eagle's medium, chicken embryo extract, foetal bovine serum and antibiotic/antimycotic mixture. Cultures were incubated at 37°C, in humid air containing 5% CO_2. Four muscle explants from each culture, removed on 10 consecutive days after incubation, were prepared for EM study. Pre-incubation specimens served as controls. A detailed experimental protocol is published elsewhere.[9]

In the first few days of culture, myonuclei underwent activation, became heterochromatic and formed into cells.[9] By the fourth day the numbers of myonuclei decreased whereas the number of cell forms within the myofibres increased. Some of the cells were fusiform and others had cytoplasmic projections or pseudopodia resembling those of macrophages (Fig. 1*A*). Some cells had phagosomes with degenerate fibrils of the parent myofibre (Fig. 1*B*). Other cells (Fig. 1*C*) displayed phagocytosis and/or pinocytosis. Many of these cells (Fig. 1*B* and *C*) with phagosomes and those displaying phagocytosis had young, developing myofibrillar elements in their cytoplasm. Electron-dense areas outlining development of Z-line material[10] were observed along many of the young myofibrils. These cells probably represented early myogenic cells or mononucleate myotubes. Many multinucleate myotubes with phagosomes, pinocytic vesicles and pseudopodia (Fig. 1*D*) were also present. All these myogenic cells were evident in cultured muscle explants after only 4 days of incubation. Sublaminar cells observed in the controls[9] morphologically represented typical satellite cells with scanty cytoplasm.

Earlier regeneration studies[3,11,12] performed *in vivo* were a problem, in that the injured myofibres rapidly underwent necrosis as a result of poor circulation due to blood clots. 'Outside-muscle' influences[13] such as migrating phagocytes

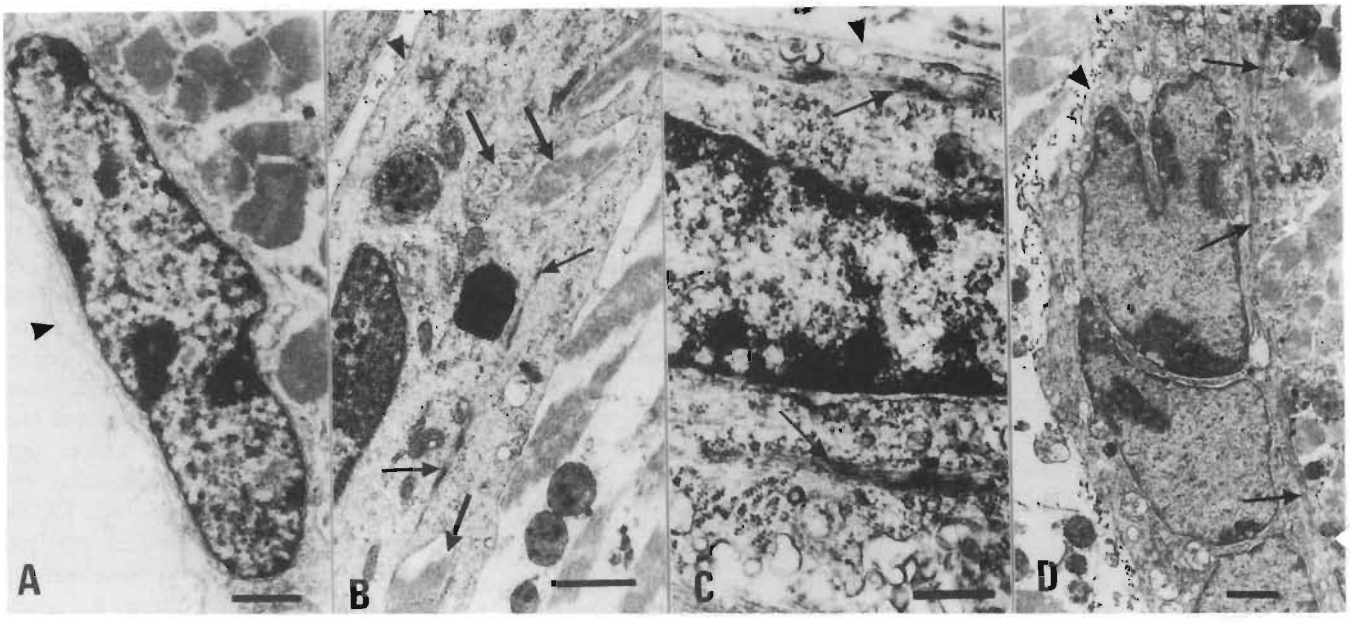

Fig. 1. Electron micrographs represent parts of sublaminar cells observed in myofibres of cultured explants. *A*, Cell, 3 days after incubation, with cytoplasmic projections/pseudopodia similar to that of a phagocyte. *B*, Part of myogenic cell mimicking a phagocyte having phagosomes (thick arrow) with degenerate fibrillar elements of the parent myofibre. Distinct developing myofibrils (thin arrow) are present in the cytoplasm. *C*, Part of another cell indicating clear signs of phagocytosis and pinocytosis together with pseudopodia. Early myofibrils (arrow) with Z-band development are present in the cytoplasm. *D*, Part of a multinucleate myogenic cell with pseudopodia intimately associated with the degenerate cytoplasmic elements of the parent myofibre. The cytoplasm of this cell has distinct developing myofibrils (arrow). All the above cells with developing myofibrils were observed in the explants from 4 days after incubation. Arrow head, basement membrane. Bar = 1 μm in *A*, *B* and *D* and 0.25 μm in *C*.

and fibroblasts made clear identification between myoblasts and other sublaminar cells difficult. The present *in vitro* technique using ME in culture overcame much of this problem. The cultures were without clots, ensuring adequate perfusion of the ME with nutrient medium. Outside elements, such as migratory or invading cells of fibroblasts and phagocytes, were minimized. This technique therefore made possible the observation of the different stages of muscle regeneration.

This study implies that cells with myogenic potential, found in a sublaminar position during regeneration of muscle, may have variable morphology, appearing as fusiform cells or as macrophages. The configuration of these sublaminar cells depends on the availability of space within the myofibre. A logical explanation for the early cells being fusiform is that the myofibres at this stage still have intact myofibrillar material such that the myoblasts could only develop sandwiched between the basement membrane and the myofibrils. As degeneration and fragmentation of myofibrils of the parent myofibre occur, space becomes available within the myofibre, allowing the myoblasts to develop cytoplasmic projections or pseudopodia. During the course of their development to myotubes, it appears as if the myoblasts engulf or phagocytose cytoplasmic elements from the degenerating parent myofibre, not so much to remove debris, but to use it as a source of nutrients for their growth. In doing this, their morphology resembles that of macrophages. Mastaglia and Walton[10] support this by suggesting that regenerating cells may 'actually reutilise breakdown products of necrotic sarcoplasm.' Therefore, the siting of similar myogenic cells, morphologically similar to macrophages in early regeneration studies, perhaps led to the suggestion[14] of the possibility of phagocytes and other connective tissue cells playing a role in muscle regeneration. Those laboratories that are set up for histochemistry and immunocytochemistry would be able to validate these findings.

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