THE UNIVERSITY OF MANITOBA

SEARCH FOR ABNORMALLY SYNTHESIZED PROTEINS IN NERVOUS TISSUES OF HAMSTERS AND MICE WITH GENETICALLY DETERMINED MUSCULAR DYSTROPHY

by

Marilyn D. Hayward

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For my friend and husband Rolly

with love-

"...let truth be free to make her sallies upon Thee, and Me, which way it pleases God. For who knows how, better than he that taught us first to plough, to guide our mind and pens for his design? And He makes base things usher in Divine...."

-John Bunyan, Pilgrims Progress

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ABSTRACT

Muscular dystrophy is a group of genetically determined diseases characterized by muscular weakness and wasting. As in other inborn errors of metabolism the genetic defect(s) in the muscular dystrophies appear to be expressed in many tissues besides muscle and heart. Assuming that the genetic defect is expressed in(an) altered protein(s) a systematic search was performed for abnormally synthesized proteins in nervous tissues of two animal models of muscular dystrophy, the genetically determined muscular dystrophies in hamster, strain BIO 14.6 and mice, strain 129Rej/dydy.

To investigate the metabolism of the two different animals(diseased and control) simultaneously and to ensure exact reproducibility between the fractionation of diseased and control animal tissues a dual labelling technique was employed. Radioisotopes of leucine, L-(U^{14}C)leucine and L-(4,5^{3}H)leucine, were injected intraperitoneally into age-matched pairs of dystrophic and normal animals respectively. After a set period of incubation with the radioisotope the animals were killed and tissues were excised, combined into one preparation for homogenization and subcellularly fractionated. The dual labelling technique used in this way allows the comparison of the in vivo incorporation of ^{3}H and ^{14}C-leucine into various protein fractions in the normal and dystrophic animals. Proteins were separated by three analytical methods: SDS gel electrophoresis, disc gel electrophoresis and isoelectricfocusing.

A study of brain subcellular fractions of Syrian hamsters(1-6-days-old) by SDS gel electrophoresis and isoelectricfocusing showed no detectable difference between normal and dystrophic hamsters. An apparent abnormality of the particulate fractions was found to be an artifact of the leucine labels and could be removed by acetone extraction.

The dual labelling technique was used to investigate known neural abnormalities of the murine dystrophy. Preliminary work with the brain soluble and particulate fractions(mitochondrial) indicated no detectable abnormality was present in the dystrophic mice(4-12-weeks-old). Extirpated spinal cord homogenates of these mice showed protein abnormalities.

Dystrophic mice which were 29-days-old showed a decreased label incorporation into protein fractions in a region of an approximate molecular weight of 23,000. This may have been a true abnormality ultimately caused by the dystrophic gene. Lubrol solubilized homogenates of the spinal cord fractionated on isoelectricfocusing gels indicated two abnormalities in dystrophic mice of both 47 and 66 days of age. SDS gels of unlabelled spinal cord, spinal roots and sciatic nerve gave reproducible differences between 129Rej/dydy and 129Rej/+? mice; the dystrophic mice showing a decreased quantity of several protein components of high and low molecular weights. Using the 4.5% polyacrylamide gel with SDS but a Tris-EDTA buffer system spinal cord homogenates showed several components altered in concentration in the dystrophic mice compared to their normal littermates.

The pattern of leucine incorporation into various protein fractions of brain from both dystrophic mice and hamsters showed a remarkable identity between normal and dystrophic animals. This can be taken as conclusive evidence that there are no gross defects in any of the more abundant proteins of the brain.

Several abnormalities were detected in the spinal roots and sciatic nerve of dystrophic mice. However, the incorporation of labelled leucine into these fractions was too low to resolve the abnormalities reliably. Future studies, possibly on newborn animals and employing two-dimensional electrophoretic techniques may be required to clarify these findings.

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LIST of ABBREVIATIONS

ACh.....acetylcholine
AChE.....acetylcholinesterase
CAT.....choline acetyltransferase
d.....daltons
DMD.....Duchenne muscular dystrophy
DNA.....deoxyribonucleic acid
EDB.....extensor digitorum brevis muscle
EDTA.....ethylene diamine tetraacetic acid
EGTA.....ethylene glycol-bis(-aminoethyl ether)N,N tetraacetic acid
EDL.....extensor digitorum longus muscle
G-6-PDH.....glucose-6-phosphate dehydrogenase
IEF gel.....isoelectricfocusing gel
HbS.....sickle cell hemoglobin
Leu .....leucine
LDH .....lactate dehydrogenase
mepps ..... miniature end plate potentials
MeV ..... million electron volts
MD ..... muscular dystrophy
MICRO ..... microsomal pellet subcellular fraction
MITO ..... mitochondrial pellet subcellular fraction
mm ..... millimetres
M.W.....molecular weight
NADH ..... nicotinamide adenine dinucleotide
N-DNP-L-LEU.dinitrophenyl-L-leucine
NP ..... nuclear pellet
PA ..... polyacrylamide
PAGE ..... polyacrylamide gel electrophoresis
PM ..... picomoles
PMSF ..... phenylmethylsulfonylfluoride
RBC ..... red blood cell
SC ..... Schwann cell(s)
SD ..... standard deviation
SDH ..... succinate dehydrogenase
SDS.....sodium dodecyl sulfate
SOL.....soleus muscle
spec. act...specific activity
SR.....sarcoplasmic reticulum
SUP.....ultrasupernatant fraction, supernatant
T.D. ..... tracking dye
TEMED.....N,N,N',N'-tetramethylethylene diamine
uF .....microfarad
ul .....microlitre

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INTRODUCTION

The muscular dystrophies are a heterogeneous group of inherited diseases in man and animals. These diseases are characterized by striated muscle weakness and necrosis and usually lack any symptoms of nervous system disease (1). The progressive nature of these diseases is shown most dramatically in the human Duchenne muscular dystrophy. The disease heterogeneity is reflected in the different histological, electrophysiological and biochemical features of each type of muscular dystrophy as well as in their mode of inheritance. Any chance that these diseases have the same primary genetic(DNA) abnormality and biochemical expression of that abnormality at the cellular level is unlikely.

For many years the primary expression of this lesion was thought to occur in the muscle itself though the DNA carrying the muscular dystrophy mutation is contained in every cell (2). In recent times, however, the nervous system has been implicated in these diseases (3-16).

Why isn't the DNA abnormality studied directly, whether studying nervous tissue or striated muscles? The DNA lesion is difficult to study directly for two reasons. The first reason is the number and composition of gene loci present in each cell. In man there are between 40,000 and 100,000 structural gene loci (17). Each gene locus contains the nucleotide base sequence which is transcribed into its corresponding RNA and subsequently translated; resulting in a protein molecule(18). For every amino acid in the protein there is a corresponding triplet of nucleotide bases at the gene locus((19). This would mean that a small protein of 100 amino acids would be coded for by a base sequence of 300 nucleotide bases. The average length of a structural gene locus, however, is between 1000 and 1500 nucleotide base pairs (20). To study the number of bases involved at all the gene loci would be a formidable task.

The second reason there is a difficulty in studying DNA directly is that a point mutation, even a single base nucleotide substitution, can result in a grossly abnormal protein (19). These point mutations may result in changes in the charge(HbS in sickle cell anemia), in the molecular weight(haptoglobin variants), in the stability of a protein (glucose-6-phosphate-dehydrogenase variants), in the rate of synthesis of

of a protein or a complete failure of protein synthesis(thalasssemias) (19). Other mutations like crossing over on homologous chromosomes, or abberrant union after breaks in chromosomes occur, may result in duplication or deletion phenomena which bring about a change in the molecular weight of the protein (19). These mutations may alter the stability of the protein resulting in an altered half-life of the protein.

These various mutations, expressed in the amino acid sequence of a protein, may result in an abnormality which can be detected on the basis of altered molecular weight or altered charge of the protein. Differing rates of synthesis or degradation may also be detected in an analytical system which detects proteins quantitatively; such as polyacrylamide gel electrophoresis with sodium dodecyl sulphate(SDS-PAGE) or polyacrylamide gel electrofocusing(IEF gel).

The dual isotopic labelling technique is useful for studying genetically dystrophic and normal tissues. Dual labelling allows the simultaneous measurement of two different radioactive isotopes in the same sample. The isotopes can be distinguished on the basis of their half-lives or their energy spectrums, and therefore the amount of incorporated label present in a particular tissue or subcellular fraction can be determined. It is assumed that no biological discrimination occurs by the tissues in the utilization of the different isotopes of leucine.

In this study age-matched pairs of normal and dystrophic animals were injected with equimolar concentrations of a radioisotope of leucine (Figure 1). The tissues from the two animals were combined, homogenized and fractionated together therefore no anomalous differences related to differential sample preparation arise. This is an advantage of the dual labelling technique.

Because many thousand proteins exist in a single cell and the functions of only about 1300 are known (21) it may prove impotent to seek out specific cell proteins for analytical study. This would be especially true in genetic diseases where the disease origin is not known. Even when the 'basic defect' or abnormality is thought to be understood, unsuspected discoveries may show our limited knowledge and/or understanding of genetic diseases. In a recent study of cultured Pompe's fibroblasts where the

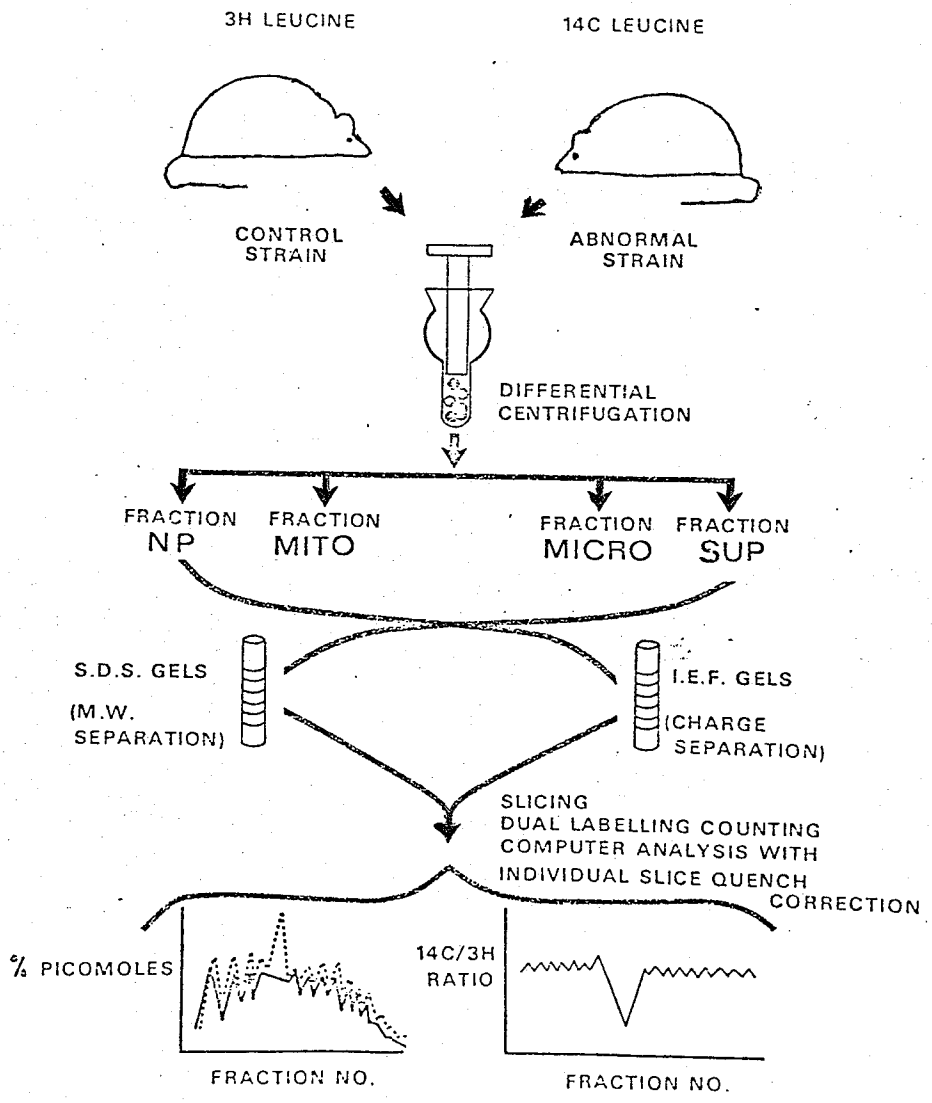

FIGURE 1. Flow scheme of the double isotopic labelling technique.

known basic genetic defect was a deficiency of α-1,4-glucosidase a second abnormality was found (22). This result was obtained using ^{3}H-and ^{14}C-leucine isotopes for labelling the Pompe's and normal fibroblasts in culture. The dual labelling technique has another advantage, then, in that it is unbiased.

This technique will be used to search for abnormal proteins of the nervous system tissues in the dystrophic hamster, strain BIO 14.6, and the murine dystrophy of Bar Harbour 129Rej/dydy mice.

HISTORICAL LITERATURE REVIEW

A. Muscular Dystrophy in Man and Animals

The muscular dystrophies are characterized by striated muscle weakness and necrosis. In the early phases of Duchenne muscular dystrophy (DMD) the patients' muscle appears to increase in mass. This is not due to an increase in muscle fibre material but to a replacement of the necrotic muscle cells by an excessive amount of fat tissue (1). Several histological changes in individual muscle fibre breakdown in DMD have been seen. These have been divided into five basic stages by Cullen and Fulthorpe (23). In Stage 1 the muscle fibres appear normal yet they have an increased volume in the sarcoplasm by 35-80%. A decrease in the mitochondrial volume also occurs. In transverse section the muscle fibres appear rounded rather than polyhedral in shape (23,24). In Stage 2 there is overcontraction of the myofibrils around the Z-line associated with overstretched regions in other parts of the sarcomere. Areas of overstretching are regions where no overlapping of the A and I bands occur. Z-line abnormalities occur and they are no longer at right angles to the longitudinal axis of the muscle fibre (23). Localized contraction continues in Stage 3 with the overstretched areas partly empty of the contractile myofibrils. The 'hyaline degeneration' characteristic of muscle fibres of DMD patients' occurs in Stage 4. As the clumps of myofibrillar elements becomes more condensed retraction clots and retraction caps form. The final stage(Stage 5) occurs with the invasion of the muscle fibre by macrophages. Areas of hypercontraction have also been seen in Duchenne patients which show plasma membrane lesions (25).

Fatty infiltration(lipomatosis) also occurs in the muscle of these patients (26) along with the formation of fibrous tissue (27). An increase in the number of subsarcolemmal nuclei with 'nuclear rowing' is seen in the muscle fibres of patients with muscular dystrophy (24).

Several animals with an inherited muscular dystrophy show similar pathological characteristics to the Duchenne muscular dystrophy and have been used as models in the study of this disease. The hamster strain BIO 14.6 has a form of muscular dystrophy which involves the heart and skeletal muscle(28). The skeletal muscle shows the nuclear rowing and central localization of the nuclei, myolysis and macrophage infiltration and some fat accumulation in the muscle mass as is found in DMD (28). The mouse(murine)dystrophy, strain Bar Harbour 129Rej/dydy, also has these histological characteristics as well as nervous system abnormalities (14, 29,30,31).

These animal forms of dystrophy are inherited in an autosomal recessive pattern in contrast to that of the Duchenne muscular dystrophy inherited in an X-linked recessive pattern (1,32,33). Another animal model for muscular dystrophy, which has some similarities to the Duchenne muscular dystrophy, is that of the domestic chicken. This muscular dystrophy is co-dominantly inherited and affects mainly the fast(glycolytic) muscle fibres of the pectoral muscles of the chicken (34). The pectoralis muscle shows major phagocytic cell infiltration and fat replacement of the muscle mass as well as vacuolization of the muscle fibres (26,35).

Though the animal forms of muscular dystrophy show similarities to DMD it is possible that the primary genetic lesion in these diseases is not the same. The study of muscular dystrophy in these animals may however

lead to an understanding of their particular biochemical abnormalities and thus the differences between dystrophic and normal animal tissue.

B. Evidence for Neurologic Involvement in Muscular Dystrophy

Because of the pathological characteristics of muscle in the muscular dystrophies they have been classified as myopathic diseases. A myopathy is usually defined as a disease with the primary disorder in the muscle (2). There are, however, several characteristics of muscle which are determined by the nerves which innervate them. The velocity of contraction and twitch duration can be altered by changing the nervous supply to the muscle (36). The enzyme and structural protein patterns of muscle can change as a result of a change in nervous stimulation; most specifically those proteins of the sarcoplasmic reticulum (37), myosin light chains in the contractile proteins(38,39) and enzymes involved in energy metabolism of muscle (40,41). The localization of acetylcholinesterase(AChE) activity to the muscle sole plate also does not occur without the nerve synapsing with the muscle fibre (42). The characteristics of muscle in DMD and those in denervated conditions or with altered neuronal influence often ressemble each other in morphology, biochemistry, and electrophysiologically. One might have expected this to lead to the investigation of nervous system involvement in muscular dystrophy and even to the possibility that the dystrophy is neurogenic rather than myogenic in origin. However, it was not until in 1967, when McComas and Mrozek found defective motor innervation of the extensor digitorum brevis (EDB) muscle in the dystrophic mouse, strain 129Rej/dydy, and in 1970 when McComas found a reduction of the number of motor units in the EDB muscle in patients with DMD, that a neurogenic origin for muscular dystrophy