A THESIS ENTITLED

STUDIES ON THE IMMUNOPATHOLOGY OF INFLAMMATORY MUSCLE DISEASE

submitted for the degree of

DOCTOR OF PHILOSOPHY

in the

Faculty of Science

University of London

by

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ABSTRACT

Inflammatory muscle disease is a rare condition which includes polymyositis and dermatomyositis. Although its aetiology is unknown, its pathogenesis, which results in damage to muscle fibres, is thought to have a lymphocyte mediated basis. Research into this desease has been handicapped by the lack of suitable *in vitro* correlate of immunologically mediated muscle cell damage.

These studies set out to establish an *in vitro* myotoxicity assay based on the culture of human fetal skeletal muscle and a radiolabel specific for myotubes (pre-muscle cells), tritium-labelled carnitine. Initially, the kinetics of carnitine transport across myotube membranes was studied in the mixed cell cultures derived from disaggregated muscle tissue. As these experiments proceeded, research was designed to examine the correlation between clinically determined disease activity and *in vitro* measurement of myo-toxicity by peripheral blood lymphocytes from affected individuals, and the value of these tests in distinguishing disease groups.

Investigation of the movement of tritium-labelled carnitine across muscle cell membranes in human fetal muscle cultures, showed that carnitine was taken up by myotubes by an active transport mechanism. The parameters of this system were established, including the Kn and Vmax of the enzymes involved. The specificity of carnitine uptake by myotubes was also demonstrated by freeze-dried autoradiography. Using human fetal skeletal muscle cultures labelled with carnitine in short term cytotoxicity assays, it was shown that peripheral blood lymphocytes from patients with polymyositis specifically damaged myotubes and that this cytoyoxic ability correlated with clinical activity of the disease. The occasional finding of myotoxicity by lymphocytes from normal individuals, led to the demonstration that a significant proportion of the cytotoxicity was mediated by Spontaneous or Natural Killer cells. Consequent measurement of Natural Killer cell activity in patients with polymyositis revealed that some specificity for muscle cell targets was evident in peripheral blood lymphocyte populaions from polymyositis patients, but not in those from normal individuals.

The clinically based experiments showed that lymphocytes from patients with polymyositis have some specificity in their *in vitro* cytotoxic responses for muscle cell antigens. The results of this thesis suggest the direction, and provide some of the methods for further investigations into the pathogenesis of inflammatory muscle disease.

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The uptake of tritium-labelled carnitine by monolayer cultures of human fetal muscle and its potential as a label in cytotoxicity studies.

Cambridge, G. and Stern, C.M.M. (1981).

Clin. exp. Immunol., 44, 211-219.

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ACKNOWLEDGEMENTS

This thesis is dedicated to the memory of Professor Tony Waterson; a friend greatly missed.

I wish to thank my supervisor, Dr Colin Stern, for his encouragement, sagacious advice and the unbelievable patience he showed during the preparation of this thesis.

Ginny Wolstencroft typed this work and I wish to thank her for her thoughtfulness as well as her technical expertise.

ABBREVIATIONS

ADCC	Antibody-dependent cytotoxic cells
ANA	Anti-nuclear antibody
BCG	Bacille Calmette-Guerin
C_2	Second component of complement
Ci	Curie
CM	Culture medium containing:
	199 Medium
	0.35% sodium bicarbonate
	5 μg/ml gentamicin
	2 mM/ml L-Glutamine
CO_2	Carbon dioxide
CPK	Creatine phosphokinase
cpm	counts per minute
^{51}Cr	51-sodium chromate
DNA	Deoxyribonucleic acid
DM	Dermatomyositis
EAM	Experimental allergic myositis
EMG	Electro-myography
E/T	Effector-to-target cell ratio
FCA	Freund's complete adjuvant
FCS	Fetal calf serum
Fcγ	Fc fragment of immunoglobulin G
FcγR	Receptor for Fc fragment of immunoglobulin G

GM	Growth medium containing:
	RPMI 1640 culture medium
	2 mg/ml sodium bicarbonate
	0.4 mg/ml L-Glutamine
	100 IU penicillin
	100 µg/ml streptomycin
	5% fetal calf serum
HBSS	Hanks' balanced salts solution
^{3}H-C	Tritium labelled carnitine
HEPES	N'-2-hydroxyethylpiperazine-N'-ethanesulphonic acid
HFF	Human fetal fibroblast
HFM	Human fetal muscle
HH	Hammersmith Hospital
HLA	Human leukocyte antigens
Ig	Immunoglobulin
IMD	Inflammatory muscle disease
IU	International units
M	Molar
mg	milligrams
MHC	Major histocompatiblity complex
min	Minutes
ml	millilitres
mM	milliMolar
MM	Maintenance medium containing:
	RPMI 1640
	2 mm L-Glutamine
	0.2% sodium bicarbonate
	10 mM HEPES

NIMD	Non-inflammatory muscle disease
NK	Natural killer
OE	Ox erythrocytes
PBL	Peripheral blood lymphocytes
PBM	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PLL	Poly-L-Lysine
PM	Polymyositis
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SLE	Systemic lupus erythematosus
Trypsin/EDTA	0.025% trypsin and 0.5 mM disodium ethylene diamine tetra-acetic acid
UCH	University College Hospital
µg	micrograms
µl	microlitres

SECTION I

INFLAMMATORY MUSCLE DISEASE: HISTORICAL REVIEW, CLINICAL FEATURES AND EXPERIMENTAL STUDIES

I INFLAMMATORY MUSCLE DISEASE: HISTORICAL REVIEW, CLINICAL FEATURES AND EXPERIMENTAL STUDIES

CHAPTER 1

I.1 HISTORICAL REVIEW

In 1863, a young German physician named Erlich Wagner described a patient with an 'acuta progressiva' generalised muscular affection with a pronounced skin rash, which advanced rapidly to a fatal outcome within 6 days. Over 20 years later, again in Germany, Hepp (1887) published the account of a similar case, although without skin involvement, calling it pseudo-trichinosis, a special form of polymyositis. In the same year, 1887, Wagner described another case of what he called 'Polymyositis', the symptoms resembling those of the patient he had previously reported. It was Unverricht (1887) who first used the term 'dermatomyositis', because of the skin involvement, concurrent with muscle weakness, in the condition he also described as 'acuta progressiva'. In 1891, Unverricht stressed the noticeable proximal distribution of muscle weakness in these patients and made the observation that spontaneous recovery could occur. The occasional involvement of other muscle tissues, namely ocular and cardiac, was first reported by Oppenheim in 1899 and 1903. All of these early publications recorded the disease as being acute in onset and progression, but Petges and Cléjat (1906) described a form of the disease with a more chronic course.

Most of the early descriptions of this clinical condition were from

Germany, although there was one reported case from the United States (Jackson, 1887). The first cases in the United Kingdom were documented by Gowers, both in 1899. This author used the term 'polymyositis' to describe the disease, despite the fact that both of the patients had overt dermal involvement. This seemed to emphasise the clinical similarity of muscle symptoms in patients either with or without skin disease and, for many years, the terms polymyositis and dermatomyositis were used interchangeably. Only in the last 30 years have the classifications most often used by physicians stated that skin involvement may be a major feature of the disease, when present, and the term dermatomyositis should be used. If it is completely absent, polymyositis is the preferred diagnostic term. However, there still seems to be much confusion when the skin rash is atypical or transient.

The first review of the literature was published in 1891 by Strumpell, who annotated 10 cases and added one of his own. By 1905, Steiner (1901-1905) was able to describe 28 cases of this 'rare muscle anomaly' which had a high mortality rate; 17 of these patients had succumbed to the disease. Karelitz and Welt reviewed the available literature on polymyositis and dermatomyositis in 1932, and estimated that 75 cases had been described and one third of these had occurred in children. Since then, there have been many reported cases; 263 reviewed by Scheurman in 1939; Domzalski and Morgan (1955) estimated that 600 patients had been described. There have been several large surveys conducted at the Mayo Clinic and at the University of California and Los Angeles in the United States (Winkleman et al, 1968 and Bohan et al, 1977, respectively) and Newcastle in the United Kingdom (Walton and

Adams, 1958; Rose and Walton, 1966; Devere and Bradley, 1975). In addition, the most commonly referred to critical reviews of diagnostic and classification criteria have been published by Bohan and Peter, (1975) and Pearson and Bohan, (1977). The most recent review is by Hudgson and Walton (1979).

CHAPTER 2

I.2 CLINICAL FEATURES

I.2.1 Definition

Polymyositis is defined as an inflammatory myopathy of unknown aetiology.

I.2.2 Signs and Symptoms

Polymyositis commonly presents in an insidious fashion, with weakness developing over weeks, months or even years. The proximal muscles of the lower limbs are usually the first affected, causing difficulty in climbing stairs and rising from a chair. However, there are several reports of unusual presentations, in which the distal muscles are predominantly affected (Eaton, 1954; Hollinrake, 1969; Van Kasteren, 1979 and Bharucha and Morgan-Hughes, 1981). Muscle pain and tenderness are found in under one half of the patients, and pharyngeal weakness with the associated symptom of dysphagia in about one fifth (Bohan and Peter, 1975). Occasionally, an acute 'nodular' presentation is found, with intensely painful muscle swellings, which may initially be confined to a single muscle or a limited number of muscles (Cumming et al, 1977; Bharucha and Morgan-Hughes, 1981). Wasting of the muscles or reflex change are not usual features of uncomplicated polymyositis (Bohan et al, 1977).

Patten (1977) observed that Raynaud's phenomenon and evidence of

arthralgia or alveolitis were found in association with polymyositis in one quarter of adult patients. Interstitial pneumonitis and gastrointestinal disturbances are rare. When the characteristic skin rash accompanies the myopathy, the term dermatomyositis is employed. When present, the dermatological features include a lilac discolouration of the eyelids (heliotrope rash), sometimes with periorbital oedema, and scaly erythematous changes, which are most frequent over the extensor surfaces of the hands and sometimes appear over the large joints, face and upper body. These features may also be seen in association with scleroderma (Dowling, 1955). Systemic features, such as lassitude, anorexia and weight loss, are relatively infrequent in polymyositis (Patten, 1977). In childhood dermatomyositis, misery and failure at school sports are usually the first symptoms and the florid skin rash commonly often appears before muscle symptoms (Krain, 1975).

I.2.3 Incidence/Genetic predisposition

Polymyositis and dermatomyositis are rare conditions with a combined reported incidence of 0.5 per 100,000 (Medsger, Dawson and Masi, 1970) and prevalence of 8 per 100,000 in Caucasians. The occurrence of adult disease is more frequent in women than in men, in the ratio of 3:1 (Devere and Bradley, 1975; Hudgson and Walton, 1979). In children, there appears to be an equal sex ratio. They may occur at any age, although, apart from childhood dermatomyositis, the mid-adult years (4th and 5th decades) are the most common. When inflammatory muscle disease is associated with a neoplasm in patients over 40, a slight preponderance of male to female was noted by Bohan and Peter (1975).

Information regarding associations between polymyositis and dermatomyositis and HLA alleles is scanty and the studies are difficult to interpret and compare because of the small numbers of patients, differences in diagnostic criteria (particularly with respect to associated connective tissue diseases and presence of inflammatory muscle disease) and different population backgrounds. However, Pachman et al (1977) reported that HLA-B_8 was over represented amongst patients with childhood dermatomyositis and Behan, Behan and Dick (1978) noted a similarly increased frequency of this allele in adult myositics. Cumming et al. (1979) found HLA-B_{14} and HLA-DR_3 to be present in a significant proportion of adult white patients. HLADw6 was shown to be over-represented amongst adult black patients by Hirsch and his collaborators in a recent study (1981).

Familial inflammatory muscle disease has been reported rarely (Howard and Thomas, 1960; Lambie and Duff, 1963; Lewkonia and Buxton, 1973).

I.2.4 Diagnosis

The diagnosis of inflammatory muscle disease, a disease group that embodies polymyositis and dermatomyositis, rests on five major criteria, although there is considerable controversy about how rigorously these criteria must be met for the diagnosis to be made in individual cases.

i Progressive muscle weakness

Affected muscles may include upper and lower limb muscles, anterior

neck flexors, abdominal and para spinal muscles with or without dysphagia, and respiratory muscles.

ii Elevation of serum enzymes

Aldolase, creatine kinase, lactate dehydrogenase (predominantly isoenzymes 2 and 3) aspartate aminotransferase (synonomously SGOT) and alanine aminotransferase (synonymously SGPT) are those most often increased.

iii Muscle biopsy changes

Necrosis and phagocytosis of muscle tissue, perifascicular atrophy, variation in fibre size and sarcolemmal nucleii with prominent nucleolii are often seen. Regeneration of fibres, as shown by basophilia, and an interstitial inflammatory infiltrate of mononuclear cells, which can also be perivascular, are other typical features.

iv Electromyographic abnormalities

Increased membrane irritability with high frequency, bizarre, repetitive discharges are typical changes.

v Dermatological changes

These may include a heliotrope rash with periorbital oedema and a scaly erythematous rash over the hands (extensor surface), which may also occur over the large joints, face and chest and upper back. This

distribution is said to be pathognomic of dermatomyositis.

These criteria, based on those devised by Bohan and Peter in 1975, are used by most clinicians and in the clinical studies described in this thesis. The confidence limits assigned to these criteria were as follows; definite = 4 or 3 + rash, probable = 3 or 2 + rash, possible = 2 or 1 + rash. In the studies described in this thesis, all the patients included were regarded as definite or probable by the clinicians in charge.

It is not within the scope of this thesis to criticise diagnostic features or the classification schemes commonly used. However, several points of interest will be mentioned. For example, the creatinine kinase (CPK) levels may be normal in up to 30% of cases (Morgan-Hughes, 1977) and have been shown to be unhelpful as a measure of disease activity in most cases of dermatomyositis in childhood (Dubowitz, 1976). In addition, the CPK is elevated in congenital dystrophies (Mastaglia and Walton, 1971). Electromyography can be 'normal' in up to 18% of cases and muscle biopsy in approximately 11% of cases (Devere and Bradley, 1975). Interstitial infiltration by mononuclear cells has been noted in some muscle biopsies from patients with fascioscapulohumoral myopathy and Duchenne muscular dystrophy (Walton and Adams, 1958; Dubowitz and Brooke, 1973), although the type of infiltrating cell differs from those in biopsies from inflammatory muscle disease (IMD) patients when appropriate phenotypic markers are used to identify lymphocyte sub-sets (Rowe et al., 1981; Rowe, Isenberg and Beverley, 1983).

I.2.5 Classification

Two major classification schemes for inflammatory muscle disease are usually referred to, being those of Walton and Adams (1958), represented in Table 1, and Bohan and Peter (1975) in Table 2. Rowland, Clark and Olarte (1977) have discussed these classifications at some length and concluded that polymyositis was a heterogeneous condition; dermatomyositis being regarded as more homogeneous. The arguments over classification will continue until more information regarding underlying disease pathogenesis become available, however there are several points which are worth briefly discussing.

The major difference between the two classifications is that Walton and Adams do not distinguish a childhood group of dermatomyositis. This form of disease, with prominent skin features, is approximately 25 times more common than 'pure' polymyositis in children, compared with a 2:1 ratio of poly - to dermatomyositis in adults (Bohan and Peter, 1975). The disease in children has been recognised for many years. Steiner (1901-05) included 2 children in his study; Rotky (1912) noted 2 children out of 40 patients. Banker and Victor (1966) argued for a distinction between the diseases occuring in adults and children. In children, the generally florid skin rash commonly appeared before muscle symptoms, although misery and failure at school sports could sometimes be noted for a variable period. Dermatological features were the most frequent presenting symptom in a recent study of 29 children with dermatomyositis at Hammersmith Hospital in Prof. V. Dubowitz's Department of Paediatrics and Neonatal medicine (personal communication). Banker and Victor (1966) emphasized the abnormalities

CLINICAL CLASSIFICATION OF INFLAMMATORY MUSCLE DISEASE

Table 1

Group 1. 'Pure' polymyositis: acute/subacute/chronic

Group 2. Polymyositis with minimal or transient skin involvement

Group 3. Dermatomyositis or myopathy associated with other autoimmune diseases (e.g.) SLE, rheumatoid arthritis, systemic sclerosis).

Group 4. Polymyositis/dermatomyositis in association with malignant disease.

after Walton and Adams (1958).

Table 2

Group 1. Primary, idiopathic polymyositis.

Group 2. Primary, idiopathic dermatomyositis.

Group 3. Dermatomyositis/polymositis associated with neoplasia.

Group 4. Childhood dermatomyositis/polymyositis associated with vasculitis.

Group 5. Polymyositis/dermatomyositis associated with collagen vascular disease.

after Bohan and Peter (1975).

of the small arteries, veins and capillaries and cellular infiltration of the adventitia and media of vessel walls in biopsies from three children studied. The perivascular and intrafascicular inflammation seen in adults with dermatomyositis is not as common in childhood dermatomyositis (Lell and Swendlow, 1977). Additionally, the clinical picture often seems to reflect a widespread necrotising vasculitis, with contractures and calcinosis and associated gastrointestinal manifestations, in children (Pachman and Cooke, 1980). Although calcinosis, in particular, is thought to relate to the duration of the disease, it may perhaps also be induced or aggravated by long term steroid therapy (Ansell, 1978; Sewell, Liyanance and Ansell, 1978). There is also an equal sex incidence in children. Carpenter et al., (1976) in agreement with Banker and Victor (1966), considered the dermatomyositis of children to be one aspect of the systemic angiopathy of childhood; the muscle damage being secondary to vessel damage by infarction. Another difference between the childhood and adult dermatomyositis syndromes is that there is no association with neoplasia in afflicted children (Sullivan et al., 1972).

Both classifications include a separate grouping of patients with either poly- or dermatomyositis and an associated neoplasm. Since Sterz (1916) first described a case of dermatomyositis accompanying carcinoma of the stomach, there has been much discussion as to whether the two were associated. In a review by Williams in 1959, it was reported that 15 per cent of 590 cases of dermatomyositis had associated malignancy. Bohan and Peter (1975) reviewed this aspect of inflammatory muscle disease. However, most authors consider that there is an association with neoplasia in men over the age of 50 years and

that long term follow up of cases is important, as occult neoplasms may become apparent months or years after the onset of inflammatory muscle disease (Rose and Walton, 1966). There is also a possible increased risk of malignancy following the various immunosuppressive therapies used to treat this and other diseases (Callen et al., 1980). The tumours which are most commonly associated with dermatomyositis are those of the lung, stomach, breast and ovaries (Williams, 1959).

There are several collagen vascular diseases in which an associated myositis may occur. These include scleroderma, systemic lupus erythematosus (SLE), Sjögren's syndrome and polyarteritis nodosa (Pearson and Bohan, 1977). Unfortunately, the presence of myositis in these patients is often not very thoroughly investigated, except symptomatically, thus the real incidence of myositis, as opposed to myalgia, is very difficult to determine. Even in cases where appropriate investigations are performed, interpretation of results are often controversial. For example, Tsokos and his colleagues (1981) recently tried to determine how many patients with SLE develop myositis, suggesting it was a feature of 18 out of 228 SLE patients. All 18 patients complained of tender, weak proximal muscles. However, only one had raised plasma creatine kinase; of the 8 who had electromyography examination, 5 were normal and one showed neuropathic change only. Muscle biopsies were performed on 11 and 5 showed inflammation with no fibre atrophy and six, fibre atrophy and no inflammation. It is thus difficult to see on what evidence 8% (18/228) of their SLE patients had myositis.

I.2.6 Differential diagnosis

Disorders that may enter into the differential diagnosis of polymyositis include adult onset limb girdle atrophy (Walton and Nattrass, 1954; Adams, 1973) fascioscapulohumoral myopathy (Rothstein, Carlson and Sumi, 1971) acquired lipid metabolism disorders (Boudin et al., 1976) and denervating disorders, endocrinopathies, particularly those involving the thyroid gland (McKeran et al., 1980), sarcoidosis (Gardener-Thorpe, 1972), carcinomatous myopathy (Rose and Walton, 1966) drug abuse (Lane and Mastaglia, 1978), post viral and immunization myalgia and myopathy (Kåss et al, 1979; Cotteril and Shapiro, 1978).

Bacterial invasion leading to suppurative myositis or syphilitic myositis produces localised muscle inflammation (Walton and Adams, 1958). In general, muscles are resistant to bacterial infection, with the exception of gas gangrene due to Clostridium welchii (Taylor and Henderson, 1972).

Parasitic muscle disease is rare in the U.K. Occasionally, trichinosis (due to Taenia solium) may be found (Jordon et al., 1975). Trypanosoma cruzii, the causative agent of Chagas' disease, can also cause inflammatory changes in muscle, mimicking the signs, symptoms and laboratory tests of polymyositis (Cossermelli et al., 1978) as can Toxoplasma gondii (Greenlee et al., 1975).

I.2.7 Viral infections affecting muscle

It was thought to be relevant to summarise those viral infections

affecting muscle, either directly, by invasion of muscle cells, or secondary to tissue destruction at another site. The signs, symptoms and pathological findings in these patients can resemble polymyositis and probably represent the most difficult exclusion in diagnosis. It is important to recognise a possible post-viral syndrome or presence of muscle infection for the reasons of treatment and follow-up.

Myalgia, which may be very severe, is found in association with many viral infections and may mimic the symptoms of an 'acute' form of polymyositis. Myositis and vasculitis have been reported following rubella vaccination (Hanissian et al., 1973), Hepatitis B virus infection (Pittsley, Shearn and Kaufman, 1982; Mihas, Kirby and Kent, 1978), Herpes zoster (Norris et al., 1976) and Influenza virus (Middleton, Alexander and Syzmanski, 1970; Dietzman et al., 1976) and are examples where the direct infection of muscle tissue by these agents has not been demonstrated, but inflammation, tenderness and pain of the muscles can be debilitating to the patient. There are four well-documented cases of the direct invasion of muscle by viruses. The best described are those caused by the Coxsackie B group of viruses, especially Coxsackie B which is the causal agent of Bornholm disease, sometimes called epidemic pleurodynia (Kibrik, 1964).

The difference between the usual course of disease resulting from direct infection of muscle tissue, or post-infection/immunization syndromes, and the disease defined clinically as polymyositis, is that the patient's symptoms resolve following elimination of the organism or in a relatively short time (days to weeks), requiring only symptomatic relief in the first case, but are progressive over a period of time in

polymyositis, usually requiring more aggresive therapy. However, the distinction between the two is not always as clear-cut as indicated here. Infectious agents and their possible role in the aetiology and pathologenesis of polymyositis are discussed in Section V.

I.2.8 Treatment

Many of the early descriptions of polymyositis reported a fatal outcome. Steiner (1901-05) describes 17 deaths occurring out of a total of 28 patients. In 1946, O'Leary and Waisman found a 50% death rate in a group of 38 untreated patients studied, however, diagnostic criteria were not given and the cause of death was ascertained in only 10 of these. It is meaningless to base discussions of survival of patients with polymyositis on these early figures because of the greatly improved medical care now available. Conversely, it is difficult to decide what contribution iatrogenic factors make to a fatal outcome in todays' treated patients. The most common causes of death in patients with polymyositis, are infection, respiratory failure and, occasionally, congestive heart failure (Devere and Bradley, 1975; Denbow et al., 1979).

For the past 30 years, most cases of polymyositis have been treated with corticosteroids, often accompanied by immuno-suppressive drugs such as azathioprine (Bradley and Walton, 1976; Niakan et al., 1980)), methotrexate (Metsger, Bohan and Goldberg, 1974) or 6-mercaptopurine (Goldstein, 1965), which are often prescribed for their 'steroid sparing' effect.

There has been much controversy about the efficacy of corticosteroid therapy. Whilst considerable success has been claimed for corticosteroids by Pearson (1963); Rose and Walton (1966); Mulder et al. (1963), and others such as Riddoch and Morgan-Hughes (1975) and Ziegler and Hamilton (1966) found very little benefit. As far as the author is aware, no properly controlled double blind study of corticosteroid therapy in polymyositis has been performed. An attempt to address this problem was made by Carpenter and his colleagues (1977), who could find no benefit in giving high doses of prednisolone (greater than 20 mg per day for at least 8 weeks) compared with low doses (10 mg per day or less) for the same period. Nevertheless, most physicians treat polymyositis in adults by prescribing 60-100 mg prednisolone daily in the first instance, gradually reducing the dose over a period of some months. One means of avoiding long-term high-dose oral prednisolone has been to use methyl prednisolone bolus infusions (1 gram on 3 successive days) followed by much lower doses of oral steroids (Fessel, 1980). The potential benefits of this form of treatment are at an early stage of evaluation.

The use of immunosuppressive drugs in association with corticosteroids remains empirical. A prospective double-blind trial of azathioprine and prednisolone, compared with placebo and prednisolone, showed no real difference in outcome, as judged by serum enzyme levels, muscle strength and muscle biopsy appearances before and three months after treatment (Bunch et al, 1980). Subsequently, Bunch (1981) followed up some of his patients for 3 years and claimed that the group given prednisolone and azathioprine had fared better with respect to their functional ability and lower daily steroid requirement. However, this

second study was not performed blind. One potentially serious side effect of giving two immunosuppresive agents is the danger of opportunistic infection, particularly septicaemia and pneumonia. It is also possible that the risk of steroid-induced gastrointestinal ulceration and perforation may be greater in childhood dermatomyositis (Rowland, Clark and Olarte, 1977) even though gastrointestinal involvement was documented before the use of steroids (Wedgewood, Cook and Cohen, 1953). Severe spinal osteoporosis, growth retardation, obesity, diabetes and calcinosis are well recognised side effects of long term steroid therapy, particularly in children (Ansell, 1978; Sewell, Liyanance and Ansell, 1978; Miller, Heckmatt and Dubowitz, 1983). In addition, there is always the risk inherent in chronic steroid administration that a steroid myopathy may make interpretation of weakness difficult (Askari, Vignos and Mushowitz, 1976).

However, despite all the uncertainty about the efficacy of corticosteroid therapy in polymyositis, most clinicians agree that it is the 'first line' treatment for children with dermatomyositis and the preferred therapy with a usually favorable outcome for adults with inflammatory muscle disease.

Other treatments have been tried with variable success and little follow-up in patients with polymyositis. Vitamin E injections were claimed to work by Killeen, Ayres and Mihan (1976) in a small study. Anti-lymphocyte globulin was shown to temporarily halt progress of the disease by Denman et al. (1979) and there have been several studies of the use of plasmapheresis in both adults and children (Brewer et al., 1980; Dau and Bennington, 1981 and Dau, 1982). Most cases showed a

transient relief of symptoms with relapse usual after cessation of treatment. There has recently been a revival in interest of total lymphoid irradiation as a possible treatment in intractable cases following a case reported by King-Engel, Lichter and Galdi (1981). Whilst an apparently dramatic response was recorded, the patient had been receiving large doses of prednisolone, methotrexate and cyclophosphamide in the months prior to irradiation. In addition, there is an admitted increased long term risk of neoplasia, notably leukemia, inherent in this treatment (Hoppe et al., 1977).

Summary

Although a favourable outcome is recorded in most cases of inflammatory muscle disease in adults and children, there is still a considerable morbidity, and, especially in children, complications such as calcinosis, contractures, severe wasting of muscles, growth retardation and endocrinopathy associated with the disease and/or available therapy. As the underlying cause and pathogenesis of inflammatory muscle disease is unknown, but suspected to be an auto-allergic, all treatment must be empirical and is usually directed at blanket immunosuppression. In addition, there is the possibility that different underlying causes may result in a similar clinical and laboratory profile; the tests currently available not being adequate to define such differences.

I.2.9 Measurement of disease activity

Although the diagnosis of polymyositis and dermatomyositis depends on a

combination of clinical, pathologic and laboratory findings, the significance of these tests, when used to measure disease activity sequentially, remains controversial. Measurements of muscle breakdown and nitrogen balance in patients are the most objective criteria but can only be performed at specialist centres.

As previously described, even at diagnosis, muscle enzymes may be within normal limits in up to 30% of cases, electromyography in 18% and muscle biopsy in 11% of cases. Once corticosteroid therapy has begun, assessment can become even more confusing. Long term or high dose corticosteroid therapy can result in a 'steroid myopathy' which can mimic the weakness of the disease (Edwards et al., 1981). Steroid myopathy has been claimed to be discernible from inflammatory muscle disease on muscle biopsy examination, by showing Type II fibre atrophy compared with degeneration of both fibre types. In practice, this distinction is not nearly so obvious (Dubowitz and Brooke, 1973). In addition, the ability of muscle to regenerate may be limited by the catabolic effects of corticosteroids in high doses (Pearson and Bohan, 1977).

It is generally considered that clinical assessment in conjunction with graded quadriceps muscle strength estimations are the most useful parameters of disease activity (Edwards et al., 1981). A brief review of the currently available investigations, together with their advantages and disadvantages for measuring disease activity in polymyositis and dermatomyositis has been included.

(i) Electromyography (EMG)

There is no evidence that the severity of the clinical condition in inflammatory muscle disease correlates with EMG findings. It appears to be of limited value prognostically or in guiding therapy (Streib et al., 1979).

(ii) Muscle biopsy

As Edwards, Wiles and Young (1980) emphasized, the changes seen in muscle biopsies are often too patchy to be representative or to correlate with the clinical features. Sampling error contributes to this problem. Riddoch and Morgan-Hughes (1975), in an extensive survey, concluded that the biopsy picture, using routine histological and histochemical stains, had little prognostic significance. Corticosteroid therapy in large doses (>60 mg/day) have shown to effect some reduction in the amount of inflammatory infiltrate, however, some patients were steroid responsive even when no inflammatory infiltrate was seen at any stage of their disease (Bunch et al., 1980). Schwarz, Slavin and Ansell (1980) did claim that, in their study of sequential needle biopsy, failure to respond to high prednisolone doses was correlated with a high percentage of fibres with internal nuclei in the initial biopsy. More recently, Rowe, Isenberg and Beverley (1983) have shown that there is a reduction in the inflammatory infiltrate and an altered ratio of surface phenotypes of inflammatory cells within biopsy specimens following therapy.

Corticosteroids are known to alter patterns of migration of

mononuclear cells and polymorphonuclear cells to the inflammatory sites (Turke and Parker, 1979). Whether this is due to increased vasomotor activity of the arteriolar bed, or a specific effect on lymphocyte recirculation patterns, is not known. Unlike mouse lymphocytes, human lymphocytes are relatively resistant to corticosteroid -mediated lysis, so it is unlikely that reduced numbers of cells in inflammatory sites following steroid therapy are due to a direct reduction in lymphocyte numbers.

(iii) Muscle enzymes

Damaged muscle cells are known to release a number of enzymes, the most specific for muscle being creatine phosphokinase (CPK). Although serum CPK levels are normal in up to 30% of patients at presentation, it has been claimed that, in cases where CPK levels are raised, serial measurements may be useful in assessing the effects of coricosteroid therapy (Devere and Bradley, 1975). However, even in other muscle wasting conditions, such as Duchenne muscular dystrophy, administration of steroids may lower the CPK level in serum, with no effect on the progress of disease (Professor V. Dubowitz, personal communication). CPK estimations are usually unhelpful in childhood dermatomyositis (Miller, Heckmatt and Dubowitz, 1983).

(iv) Metabolic studies and 3-methyl-histidine excretion

Metabolic studies, based on the measurement of nitrogen balance in the patient, have been carried out on several patients at University College Hospital in the Department of Human Metabolism. In these

patients, the excretion of 3-methyl-histidine, a major component of myofibrillar protein, was measured as an index of muscle breakdown. However, it was found that even when nitrogen balance became positive, urinary 3-methyl-histidine excretion remained elevated for some time afterwards, when there was net synthesis of muscle (Rennie et al., 1980).

Urinary creatinine excretion has been found to correlate with the degree of clinically determined muscle weakness in one study by Askari, Vignos and Mushowitz (1976). The main problems were related to the difficulty of ensuring that complete 24 hour urine samples were collected.

(v) Myoglobinaemia/myoglobinurea

Myoglobin, a haem-containing protein, is normally found only in striated muscle cells. The appearance of myoglobin in serum and urine has been measured in a wide variety of muscle injuries. Kagan (1977) reported myoglobinaemia in 12 out of 23 patients with poly- or dermatomyositis. Unfortunately, serum myoglobin concentration fell rapidly when steroid therapy was initiated and so has little value as a measure of disease activity, although Nishikai and Reichlin (1977) found that serum myoglobin was a more reliable indicator of the presence of inflammatory muscle disease than elevation of the creatine phosphokinase enzyme.

The urinary threshold for myoglobin is relativley high (Penn, 1982) so that searching for myoglobinuria is of little value.

In conclusion, the currently available tests of disease activity in patients with inflammatory muscle disease are hampered by their subjectivity, as in the case of clinical assessment and muscle biopsy or by the effect of therapy on their value, probably independently of any effect of therapy on the disease process causing muscle damage. A measure of disease activity more closely related to the pathogenesis of the disease would obviously be the most desirable.

CHAPTER 3

I.3 AETIOLOGY

I.3.1 Introduction

There is a body of circumstantial evidence in favour of an infective aetiology in polymyositis and dermatomyositis. Many patients at presentation complain of having suffered from a mild, febrile illness before onset of muscle symptoms. As previously discussed in Section 1, Chapter 2.3., a severe myalgia may follow infection with a number of viral agents and after vaccination or immunization. The biopsy picture in patients diagnosed as having inflammatory muscle disease resembles that seen in cases of acute viral or microbial infection (Middleton, Alexander and Szymanski, 1970). However there are a limited number of ways in which muscle can respond to insult. For example, interstitial inflammatory infiltrates and phagocytic cells are sometimes seen in nutritional myopathies, congenital dystrophies and fascioscapulohumoral myopathy.

As the finding of an aetiological agent would greatly advance studies of the pathogenesis of inflammatory muscle disease, it is not surprising that considerable effort has gone into attempts to a) isolate an infectious agent from affected muscle b) examine muscle biopsy specimens for evidence of an aetiological agent and c) measure serum antibody titres to various microorganisms. Attempts have also been made to associate the onset of muscle disease with other insults.

I.3.2 Isolation of microorganisms

There have been only four reports of the successful culture of an infectious agent from patients clinically diagnosed as having poly- or dermatomyositis. Zweymuller (1953) cultured a Coxsackie B group virus from a child with dermatomyositis. The first isolation of this virus from human tissue had been reported only one year earlier by Freudenberg, Roulet and Nicole (1952) from a nine month old child with a previously unsuspected myositis and congenital fracture of the femoral epiphysis. After this encouraging beginning, only a Coxsackie A9 virus (Tang et al, 1975) from a 9 year old child with chronic myositis who had also been dyskinetic since early infancy, an Influenza B/Lee virus (Gamboa et al., 1979) from an adult with polymyositis and an Influenza A/Texas/1/77 (Kessler et al., 1981), again from an adult with polymyositis, have been successfully cultured from muscle tissue. There are several reports of the isolation of Coxsackie B group and Influenza A and B viruses from the stools and throats of affected patients (Schiraldi and Iandolo, 1978; DiBona and Morens, 1977; Dietzman et al., 1976).

I.3.3 Biopsy examination

Much more plentiful have been reports of 'virus-like' inclusions bodies in both the nucleus and sarcoplasm of muscle biopsies. These have been of two main types. Firstly, intranuclear and intracytoplasmic inclusions composed of microtubular filaments (Chou, 1967; Jerusalem et al, 1972; Carpenter et al., 1970; Sato, 1970; Sato et al, 1971; Yinius and Samaha, 1971) which resembled those seen

in myxovirus infected cells (Morgan, Rose and Moore, 1956) have been described. Chou (1968) reported that serial muscle biopsies (taken at intervals of up to 18 months) from three of his polymyositis patients, repeatedly contained such microtubular aggregates.

There have been many reports of picorna-virus-like particles in muscle biopsies from polymyositis patients, sometimes tentatively identified on serological examination in some cases (Ben-Bassat and Machtey, 1972; Chou and Gutman, 1970; Mastaglia and Walton, 1970; Fukuyama, Ando and Yokoto, 1977). The diameter and arrangement of these particles, is approximately 23 nm with a 'crystalline' array-like appearance and closely resembles that of glycogen in degenerating muscle, so that the interpretation of these reports must include a critical review of the counterstaining methods used.

Chou (1972) compared the clinical and pathologic characteristics of a small group of patients with both types of virus-like inclusions in their biopsies. He concluded that the course of disease was more chronic in those with myxovirus-like particles, dysphagia, progressive weakness and wasting of the proximal muscles being the prominant clinical features. Myalgia, stiffness and swelling of the muscles and skin involvement were associated with proximal muscle weakness in those patients with picorna-virus inclusions. The histopathological features in these cases and in the other studies were all in general conformity with those described in polymyositis patients <u>without</u> inclusions seen in their muscle cells.

In addition, membrane-bound, intracytoplasmic aggregates have been

described in the endothelial cells of intramuscular or intradermal vessels in several cases of dermatomyositis (Norton, Velayos and Robinson, 1970; Hashimoto et al., 1971).

I.3.4 Serological studies

There have been several epidemiological studies designed to investigate whether raised antibody titres to specific microorganisms are present in the serum of patients with polymyositis and dermatomyositis.

The finding of raised antibody titres against a particular agent, compared with the expected population values, constitutes a 'low-order' association with a disease. If a four fold rise in antibody titre occurs close to the onset of disease, the association is regarded as moderate (Cambridge et al., 1979). That both low- and moderate-order associations exist between Coxsackie B group viruses and Toxoplasma gondii has been claimed for poly- and dermatomyositis. In Phillips and colleagues study (1979) of the incidence Toxoplasma gondii (Kagan, Kimball and Christian, 1974) antibodies in polymyositis, approximately 50% of patients had a higher frequency of the complement-fixing antibody, said to be associated with recent infection. The dermatomyositis patient group and those with myositis and associated connective tissue disease, did not have increased titres compared with their matched controls.

Coxsackie A and B viruses are probably the most myotropic of human viruses and are estimated to cause the majority of acute myositis and myocarditis in non-third world countries (Grist and Bell, 1974). In a

small study of four polymyositis patients, Travers and colleagues (1977) found serological evidence of Coxsackie B virus infection close to the onset of symptoms. Christiansen et al. (1983) have recently shown significantly raised neutralising antibody titres to these viruses in children with dermatomyositis. Coxsackie B group viruses are ubiquitous - most individuals acquiring antibodies shortly after starting school and having low titres of neutralising antibody in their serum for the rest of their lives. Most infections with these viruses tend to be sub-clinical, so that interpretation of data about antibody titres to these viruses must be interpreted with care and adequate control groups should be included.

Although the role of viruses in the aetiology and pathogenesis of inflammatory muscle disease is unknown, it is possible that these observations could simply reflect the ability of these viruses to behave as opportunistic passengers in muscle already damaged by the disease. They could also alter the course of the disease by increasing muscle cell damage already begun. There is some evidence to support this hypothesis. Experimental denervation or drug-induced damage of muscle leads to increased infection of that tissue by influenza virus (Hays and Gamboa, 1977; Gamboa et al., 1979). Coxsackie B virus-induced myositis in mice is significantly exacerbated in animals subjected to trauma, stress and starvation prior to or following infection (Woodruff, 1970; Gatmaitan, Chason and Lerner, 1970).

I.3.5 Other aetiological factors

There are a number of other possible aetiological factors which have

been linked with the subsequent development of inflammatory muscle disease. Drug-induced myositis following the administration of penicillin (Lane and Mastaglia, 1978) penicillamine (Petersen et al; 1978), sulphonamides (Sheard, 1971) and cimetidine (Watson et al., 1983). Symptoms usually resolve following withdrawal of the drug. There are several well documented reports of dermatomyositis developing following BCG vaccination (Kåss et al, 1979) where the disease has been apparent for years after this insult. Rubella infection (Landry and Winkelmann, 1972) and triple vaccine (Cotteril and Shapiro, 1978) immunization has also resulted in the signs and symptoms of dermatomyositis in several cases of the clinically defined disease.

I.3.6 Conclusions

Although there does seem to be some evidence for potential aetiological associations, in most patients with polymyositis and dermatomyositis, no such predisposing factor is present and all investigations are unhelpful. While it is possible that a sub-group of cases are the result of chronic infection of the muscle, and investigations are not rigorously applied or hampered by technical differences, evidence for a definite aetiological agent being involved in inflammatory muscle disease is rare.

CHAPTER 4

I.4 PATHOGENESIS

I.4.1 Introduction

The possibility exists that an infectious agent or other irritating factor may initiate an auto-allergic immune response to skeletal muscle, or other connective tissue component, in inflammatory muscle disease.

There is some evidence for disordered immune function in these patients. Additionally, there is the oft-quoted association with connective tissue diseases, including SLE, in which an aberrent immune response is known to be involved in the pathogenesis of the disease.

Complement and immunoglobulin levels are normal in most patients (Behan and Behan, 1977). Hypergammaglobulinaemia, if present, is usually mild (Lisak and Zweiman, 1976), although there are several claims that it is a feature of the disease. However, the references on which this claim rests are usually concerned with studies of patients presenting with an associated tumour: in some instances, a myeloma (Zilko and Dawkins, 1975; Tellerman-Toppet et al, 1982). There are also several accounts of hypogammaglobulinaemia, particularly associated with dermatomyositis (Giuliano, 1974; Dawkins and Zilko, 1975; Gotoff, Smith and Sugar, 1972). Specific IgA deficiency, in conjunction with picorna-virus infection, has been associated with childhood dermatomyositis (Kersey, Spector and Good (1973); Valente et al (1974); Bardelas et al (1977);

Webster et al (1978). One report of C2 deficiency, in conjunction with inflammatory muscle disease, exists (Leddy et al., 1975). Despite the numerous reports of autoantibodies and immune complexes being found in sera from polymyositis patients, the tissue damage seen in this disease does not usually reflect a humorally-mediated pathogenesis (Behan, Barkas and Behan, 1982). Vasculitis is not often a feature of adult cases of inflammatory muscle disease. Immune complex mediated renal disease and cerebral vasculitis are rare complications of this disease, although there are a few isolated cases (Pearson and Bohan, 1977). There is, however, a considerable body of evidence supporting the hypothesis that vasculitis is involved more directly in the dermatomyositis of childhood (Pachman and Cooke, 1980). Widespread tissue involvement and necrosis, particularly of the skin, is often the most important clinical problem in this disease. The gastrointestinal tract may also be involved. Although weakness of the muscles is also present, it is particularly difficult to tell whether this is due to actual inflammatory muscle disease, or as a consequence of the drug therapy to control other features of disease. Many clinicians regard the dermatomyositis of childhood as a separate syndrome, especially after the early acute phase of the disease.

Most clinical problems in adult polymyositis and dermatomyositis patients arise from muscle destruction and weakness, particularly if the respiratory muscles become involved, and any hypothesis concerning the aetiology and pathogenesis of this disease, especially in adults, must take into account this specificity for skeletal muscle.

The demonstration that delayed hypersensitivity to muscle cell antigens

can be induced and mediate experimental allergic myositis in animals, and of cellular immune responses against muscle in vitro by lymphocytes from polymyositis patients has favoured a cell-mediated pathogenesis for this disease. A muscle cell antigen which is normally present, altered or induced could serve as a recognition structure for such a response.

I.4.2 Investigations

i) Serological studies

Investigations have included the measurement of immunological parameters in serum, as well as attempts to detect specific humoral and cellular responses against cell antigens. There have been recent claims that complement activation and circulating immune complexes occur in 70% of polymyositis patients (Behan, Barkas and Behan, 1982). Immune complexes (containing IgM, IgG sometimes IgA and the third component of complement, C_3) are frequently seen in the small blood vessels of skeletal muscle biopsy specimens from patients with polymyositis, but most often in children with dermatomyositis (Whitaker and Engel, 1972; Oxenhandler et al., 1977; Ringel et al., 1979; Fulthorpe and Hudgson, 1982; Singsen et al., 1977; Alexander, Croker and Bossen, 1982). Interestingly, in the Whitaker and Engel study, the authors also found evidence of severe damage to the blood vessels in the vicinity of mononuclear cell infiltration, in the absence of immune complex deposition.

The prevalence of anti-nuclear antibodies in this group of patients

varies considerably from paper to paper; ranging from 16 (Bohan et al., 1977) to 60 per cent of patients (Venables, Mumford and Maini, 1981). A recent estimate by Reichlin and Arnett (1983) put the figure at 74%, using the more sensitive HeLa cell assay.

Additionally, there have been several claims for the specificity of antibodies against RNA-associated proteins for myositis. These have been detected using tissue extracts from several sources, by immunodiffusion and immunoprecipitation techniques. The first described specific marker for polymyositis, called PM-1 by Wolfe, Adelstein and Sharp (1977), was subsequently found to have considerable overlap with other connective tissue disease; the antibody being particularly prevalent in sera from scleroderma patients. As Nishikai and Reichlin stress in their papers (1980i and ii), there is considerable heterogeneity in the distribution of anti-nuclear and other autoantibodies; the conditions used for the immuno-diffusion and precipitation techniques contributing to the differing descriptions of antibodies found in different laboratories. There is also confusion regarding nomenclature. Nishikai and Reichlin (1980ii), describe the Jo-1 antigen, purified from calf thymus as a nuclear protein of molecular weight 150 kilodaltons. Matthews and Bernstein (1983) have shown that the antigen 'which is identical to the Jo-1 system', extracted from HeLa cells, was an RNA-associated cytoplasmic protein of molecular weight 55 kilodaltons. Although these authors do point out that this discrepancy was probably due to the different sources used to obtain their antigen, it is, nevertheless, misleading. The identity of Jo-1 antigen has been characterised at the molecular level by Matthews and Bernstein, and appears to be a sub-unit of histidine t-RNA

synthetase. However, not all patients with inflammatory muscle disease have serum antibody to this antigen. Most children with dermatomyositis do not. A large survey designed to establish the prevalence of Jo-1 antibody in myositis patients is currently underway (Dr R. Bernstein - personal communication).

Anti-muscle antibody detected by a variety of techniques, are found in a proportion of patients with polymyositis and dermatomyositis (particularly those with an associated neoplasm). These include antibodies which bind to the striations of muscle sections or to muscle specific proteins such as myosin or myoglobin (Caspary, Gubbay and Stern, 1964; Stern, Rose and Jacobs, 1967; Fessel and Raas, 1968; Nishikai and Homma, 1972 and 1977). However, such antibodies were also present in a number of myogenic and neurogenic disorders. Dawkins and Mastaglia (1973) also showed that serum from patients with polymyositis was not cytotoxic to cultured chick muscle in the presence of complement. This was a similar finding to those in experimental allergic myositis, where serum was found to be ineffective as a passive mediator of the disease and to have no myotoxic activity (Dawkins, Eghtedari and Holborow, 1971).

ii) Cellular responses to muscle cell antigens

This study of *in vitro* cellular immune responses to muscle cell antigens and cells has been claimed to provide evidence for delayed type hypersensitivity and lymphocyte-mediated damage as the most likely pathogenesis of inflammatory muscle disease. This may be so. However, a re-evaluation of these studies shows that these *in vitro* experiments

have severe limitations, both of interpretation and on technical grounds. Lymphocytes from patients with inflammatory muscle disease, have been reported to undergo blast transformation (Saunders, Knowles, and Currie 1969; Currie et al., 1971; Esiri, MacLennan and Hazelman, 1973) to produce macrophage migration inhibition factor (Caspary, Currie and Field, 1971) and lymphotoxin (Johnson, Fink and Ziff, 1972) in response to autologous and heterologous muscle and are also said to exhibit cytotoxic activity against cultured muscle (Dawkins and Mastaglia, 1973; Haas and Arnason, 1974; Johnson, Fink and Ziff, 1972). When blast transformation, following incubation with skeletal muscle homogenate, was measured, lymphocytes from polymyositis patients did show specificity in their proliferative response to muscle, as opposed to collagen, liver, kidney or encephalitic factor. The magnitude of proliferation was correlated with disease activity in the papers by Currie and colleagues (1971) and Esiri, MacLennan and Hazelman (1973). In the latter work, however, a lymphocyte transformation response to muscle cell homogenate was not confined to lymphocytes from polymyositis patients. Those from patients suffering from polymyalgia rheumatica, but not muscular dystrophy or rheumatoid arthritis (RA), showed a similar degree of transformation and correlation with disease activity. These results were not adequately explained, as the pathogenesis of polymyalgia rheumatica has not previously been thought to involve a delayed type hypersensitivity response to muscle. However, the 'negative responses' of the other controls (muscular dystrophy and RA), in which muscle degeneration does occur, suggested that the transformation response was not merely the consequence of muscle damage in vivo, but reflected the presence of specifically sensitised lymphocytes, which were capable of undergoing

secondary stimulation *in vitro* by the relevant antigens present in muscle tissue homogenates.

As well as the ability to induce a proliferative response by incubation with muscle cell antigens, Caspary, Currie and Field, (1971) and Johnson, Fink and Ziff (1972) demonstrated that lymphocytes from polymyositis patients could produce lymphotoxins. In the latter study, lymphotoxin production correlated with presence and activity of disease; surgical patients without evidence of inflammatory muscle disease were used as controls and their lymphocytes did not respond in this way. The partially- characterised lymphotoxin inhibited the uptake of radio-labelled amino-acids into fetal muscle cells. The significance of this lymphotoxin and of the macrophage migration inhibition factor described by Caspary, Currie and Field, 1974 (as having a similar pattern of production and correlation with disease activity as lymphotoxin), and their relevence to the pathogenesis of inflamatory muscle disease, has not been further evaluated. However, neither of these lymphokines were shown actually to damage cultured muscle. In addition, a number of substances released from peripheral blood mononuclear cells following antigenic or mitogenic stimulation, could exert a similar effect on the protein metabolism of cultured cells as the putative lymphotoxin reported here. Interferons, for example, are a well characterised group of molecules, known to be released from peripheral blood cells, which have a dramatic down-regulation effect on protein metabolism (Epstein, 1979).

iii) Cellular responses to cultured muscle cells

In other experiments, attempts were made to examine the possibility that lymphocytes from patients with inflammatory muscle disease could directly damage muscle cells. The *in vitro* techniques applied to try and answer this question, involved those which measured lymphocyte mediated cytotoxicity against cultured skeletal muscle. A major difficulty encountered in all those studies which employed cultures derived from explants, or which required enzymic digestion of muscle tissue, was that fibroblasts made up a large proportion of the resulting outgrowth of cells. Thus, specificity for muscle, as opposed to fibroblast, damage was difficult to determine.

In 1971, Currie and colleagues, incubated peripheral blood mononuclear cells from patients with polymyositis, with explants of human or rat fetal muscle. The patients were divided into those with 'uncomplicated' polymyositis (Type α) and polymyositis in association with connective tissue disease (Type β). Patients with systemic sclerosis were also included in these studies: however, normal individuals were not tested. The protocol of these experiments involved adding 'a drop containing between 2.5 and 5 $\times 10^5$ mononuclear cells' and with a variable number of polymorphonuclear cells, to cultures of fetal muscle or epithelial/fibroblast mixtures and observing damage to these cultures microscopically, after between two and seven days. Results indicated that mononuclear cells from patients with Type α disease were cytotoxic to muscle cultures preferentially, and those with Type β polymyositis or those suffering from systemic sclerosis were cytotoxic against both types of culture. The presence

of myotoxic activity correlated with significant values in the muscle cell homogenate lymphocyte proliferation assay, which was, in turn, correlated with activity of inflammatory muscle disease and the response to corticosteroid therapy in the patient. In addition, sera from all patients tested were innocuous to muscle and epithelial/fibroblast cultures. Apart from being interesting observations, these experiments did not allow objective quantitation of *in vitro* muscle cell damage. Dawkins and Mastaglia (1973) used ^{51}Cr labelled chick muscle, grown in roller tubes, as targets in cytotoxicity experiments designed to quantitate *in vitro* myotoxicity. As specificity controls, these researchers used chick lung fibroblasts also labelled with ^{51}Cr as alternative target cells. Peripheral blood mononuclear cells (1×10^6 per culture tube) were incubated with the labelled cultures for 18 hours. It was found that five out of nine patients with active disease showed significantly increased ^{51}Cr release from muscle cultures; compared with forty-one control individuals, consisting of 8 healthy volunteers and 33 hospital in-patients with conditions ranging from rheumatoid arthritis to myocarditis. There was also a positive relationship between clinical disease activity and percentage ^{51}Cr release from cultured chick muscle cells; cortico-steroid therapy decreased the *in vitro* myotoxicity response. A slight, but not statistically significant increase of cytotoxic activity against chick lung fibroblasts by peripheral blood mononuclear cells from patients with active disease was also reported, but the results implied a relative specificity for muscle cell targets, compared with controls. As with the studies reported by Currie et al., (1971), the results are of interest, however interpretation is limited by technical considerations. As fibroblasts

in the cultures also take up ^{51}Cr, the proportion taken up by muscle cells is limited. Friedlander and Fischman (1979) showed that fibroblasts, which comprised 10-20% of muscle cultures, contained 40-60% of the ^{51}Cr retained by the monolayer cultures. Consequently, when muscle cell damage is greatest, there is only a small difference between non-specific and specific release with respect to muscle cell killing, but a much larger difference between specific release and maximum release.

In another attempt to estimate in vitro myotoxicity, both objectively and quantitatively, Haas and Arnason (1974), approached the problem by measuring creatine phosphokinase (CPK) release from cultured fetal rat muscle as an index of muscle cell damage. As in Currie and colleagues experiments, Maximov chambers were used as the culture vessels. Peripheral blood mononuclear cells (approximately 1×10^6) were added to the cultures, and after 6 days, both CPK release into the supernatant and destruction of muscle cell monolayers was estimated. It was claimed that 9 out of 16 polymyositis patients had peripheral blood mononuclear cells capable of causing significantly elevated release of the enzyme and destruction of muscle cell cultures. However, there was no correlation between CPK release and activity or quiescence of disease; or with treatment of the patients with corticosteroids. Fifteen control patients, including 4 with myasthenia gravis, 4 with muscular dystrophy and several with neurological disorders, did not cause significant damage to muscle monolayers, as measured by CPK release.

In 1980, Haas attempted to repeat the experiments using CPK release

from rat muscle cultures as an index of *in vitro* damage in a series of 10 patients with polymyositis and 22 controls. Adding between 1 and 6 x 10^6 lymphocytes per culture, compared with 'approximately 1 x 10^6 ' in his earlier work, he claimed that no significant difference between patient and control groups was detectable in terms of CPK release into muscle culture supernatants after 6 days incubation. This discepancy with his previously reported results was suggested by him to be due to the addition of a new Zeiss spectrophotometer (which was used to measure CPK enzyme levels) to the laboratory, to replace the 'old Beckman-Guilford' machine which had been used for the earlier CPK estimations. In the discussion of this paper, Haas also discussed the phenomenon of the restriction of T-cell mediated cytotoxicity (Zinkernagel and Doherty, 1979) by products of the major histocompatability complex of both effector lymphocytes and target cells and implied that, as allogeneic muscle target cells had been used in his and other myotoxicity studies, it was not possible for there to be cytotoxicity against muscle cells targets unless syngeneic muscle was used, ignoring the phenomenon of natural or spontaneous cytotoxicity (Herberman, Nunn and Lavrin, 1975).

I.4.3 Conclusions

In conclusion, assays of *in vitro* muscle cell damage have been hampered by the lack of specificity for muscle and consequently, difficulties of quantitation. The origin of muscle cultures used as targets, and the constitution of effector cell populations, differ between investigators. In addition, dose-response relationships, calculated on a per lymphocyte basis, were not attempted in any of these studies and

it was impossible to determine the number of actual lymphocytes used in experiments, as monocytes (in effector populations) were not distinguished. Comparisons between studies were further confused, because the clinical criteria on which patient groups were chosen, and the selection of control individuals, differed greatly.

CHAPTER 5

I.5 ANIMAL MODELS OF INFLAMMATORY MUSCLE DISEASE

I.5.1 Description of animal models

There have been a number of attempts to produce an inflammatory muscle disease in experimental animals which might serve as a model for polymyositis in humans. Pearson (1956) was the first to report his studies, based on an attempt to support the speculation of the time that polymyositis had an immunopathogenic aetiology. This suggestion was made because there seemed to be a close association between polymyositis, rheumatoid arthritis and systemic lupus erythematosus, in which 'disordered immunity' was strongly suspected.

Pearson (1965) injected rabbit muscle homogenate, emulsified in Freund's complete adjuvant (FCA), intramuscularly into rats. The results were not very encouraging. Injections of FCA alone caused a similar degree of myositis, especially near the site of injection, as the muscle/adjuvant preparations. The main feature found in recipient animals of both groups was a severe arthritis.

Later experiments, again using rats as the experimental animal, were more successful (Kakulas, 1966; Currie, 1971; Esiri and MacLennan, 1974). The animals were also given homogenised rabbit muscle in FCA; in these experiments by the intradermal route, at weekly intervals. Morgan, Peter and Newbould, (1971) obtained similar results by injecting inguinal lymph nodes with autologous rat muscle. The

histological lesions produced in the muscle of these animals resembled, to some extent, those seen in patients with inflammatory muscle disease; consisting of focal segmental necrosis with an accompanying mononuclear inflammatory infiltration around the affected fibres. Regeneration of muscle, as indicated by large, vesicular nuclei and basophyllic sarcoplasm was also apparent. However, the basement membrane usually remained intact and sequelae did not include atrophy or fibrosis, which is often a feature in muscle biopsy specimens from polymyositis and dematomyositis patients. Other tissues, given in FCA, produced a mild myositis in some animals, but were not as severe as the animals were challenged with muscle in FCA. Manghani, Partridge and Sloper (1974) produced myositic changes in the skeletal muscle of guinea pigs, using similar injection regimes.

The establishment of experimental allergic myositis, or EAM, as the disease in animals was called, allowed investigations to show that afflicted animals developed a cutaneous delayed hypersensitivity reaction to muscle and not to other tissues (Dawkins, 1965; Webb, 1970). In addition, the disease was able to be passively transferred by washed lymphocytes, not serum, from affected animals to others of the same inbred strain (Esiri and MacLennan, 1974). The creatine phosphokinase enzyme in serum from such animals, was also shown to correlate with histologically-determined severity to muscle damage. Although tissue specific (anti-muscle) antibody was found to high titre in EAM animals, there was no correlation between antibody titre and the presence or severity of myositis (Dawkins, Eghtedari and Holborow, 1971). Dawkins (1965) and Dawkins and Lamont (1971) also showed that, although antibodies elicited during EAM did bind to cultured muscle,

they were not directly cytotoxic. Other *in vitro* tests indicated that the pathogenesis of the disease was mediated by lymphocytes sensitised against muscle cell antigens, and anti-muscle antibody was just a by-stander manifestation of immunization with muscle tissue. Lymphocytes from animals with EAM were able to destroy cultures of autologous fetal muscle *in vitro*, whereas those given other tissues in FCA were not, (Kakulas, 1966; Currie, 1971). Smith and Partridge (1976) further demonstrated that macrophage migration inhibition factor was produced after incubation of lymphocytes with muscle homogenate, particularly myosin and tropomyosin fractions, and not with troponin or extracts from other organs. Partridge and Smith (1976) in another series of experiments on their guinea pig model, demonstrated that lymphocytes, taken from myositic animals, specifically adhered to myotubes (pre-muscle cells), as opposed to fibroblasts also present in cultures derived from disaggregated chick muscle. Additionally, they calculated the degree of preference for lymphocytes to adhere to myotubes and found that the preferential attachment index, which they defined in their paper, correlated with the histologically determined degree of myositis in the animals.

I.5.2 Conclusions from EAM studies

The autoimmune nature of experimental allergic myositis was thus established by several criteria.

a) The induction of the disease was associated with delayed hypersensitivity and antibody production directed against the specificity of the injected material (in this case skeletal muscle).

b) The tissue damage which followed the injection of muscle in FCA was restricted to muscle tissue. The occasional finding of an arthritis, particularly in the rat studies following injection of FCA alone has been shown to be due to a component of the mycobacterium present in the adjuvant preparation, and not to defined self-antigens (Iizuka and Chang, 1982).

c) Washed lymphocytes from affected animals could transfer the disease to normal recipients of the same inbred strain.

Further, in EAM, the disease was probably mediated by cellular, as opposed to humoral, factors as shown by the presence of the in vivo delayed hypersensitivity type response to muscle antigens. In vitro experiments showing that the severity of the disease correlated with lymphocyte-mediated sensitivity and cytoxicity to muscle, not with antibody production or cytotoxic ability, further strengthened this hypothesis.

I.5.3 Comparison of EAM and human inflammatory muscle disease

There are, however, several major differences between the experimental allergic myositis produced by immunological means in animals and the polymyositis syndrome in humans. To summarise:

a) The disease in animals is self-limiting; being maintained only by repeated injections of muscle and adjuvant. After a number of injections, many of the animals became resistent to further induction of symptoms.

b) The lesions in the muscle, although histologically resembling those seen in many cases of inflammatory muscle disease in humans, failed to cause any signs of weakness in the animals.

c) EAM did not follow a course of relapse and remission. The animals recovered when no longer rechallenged with antigen and adjuvant. This contrasts with the characteristic clinical course of the illness in most polymyositis and dematomyositis patients.

More recently, Strongwater and Schnitzer (1983), have described a Coxsackie B virus-induced murine model of polymyositis. The virus could not be recovered from tissues two weeks after innoculation into neonatal mice, but electro-myographic and histological abnormalities, akin to those of human polymyositis, persisted far up to three months. Unfortunately, the experimental details of this work have not yet been published in full; the above reference alluding to an abstract. However, there are several murine models of a chronic disease of cardiac muscle, using Coxsackie B virus as the initiating organism (Reyes, et al., 1981; Huber et al., 1981). Studies of the immune response elicited and sequelae in affected tissues following experimental infection with this myotropic group of viruses have already indicated that further investigations are strongly indicated, with particular reference to chronic inflammatory muscle disease.

CHAPTER 6

I.6 INTRODUCTION TO EXPERIMENTAL STUDIES

The following studies were undertaken to develop an *in vitro* assay for the measurement of immunologically mediated damage to cultured muscle cells, in order to overcome some of the shortcomings of previous tests used for this purpose in studies on the pathogenesis of polymyositis and dermatomyositis. Despite the problems of the methods used by these researchers, measurement of disease activity in these patients did seem to correlate with *in vitro* lymphocyte-mediated myotoxicity and it was thought worthwhile to pursue investigation for reasons of both clinical and possibly aetological, or pathogenetic relevance.

As Hammersmith Hospital is a referral centre for children with muscle disorders and the Department of Rheumatology has an interest in inflammatory muscle disease, it was hoped that the co-operation of clinicians could be readily sought, if and when a suitable assay was developed.

There are several ways in which one could approach the problem of developing a specific method to measure *in vitro* myotoxicity. Firstly, one might eliminate the fibroblasts from cultures derived from muscle tissue. Several methods have previously been tried as means of reducing the numbers of fibroblasts present in these cultures, such as pre-plating (Yaffe, 1968) or the addition of cytotoxic drugs to the primary cell cultures (Moss et al., 1978) in order preferentially to destroy the more rapidly dividing fibroblasts. X-irradiation has also

been used for the same reason (Friedlander, Beyer and Fischman, 1978). These techniques do reduce the numbers of fibroblasts considerably, but elimination has not been achieved.

An alternative approach would be to employ a compound selectively taken up by the myotubes or pre-muscle cells in these mixed cell cultures. The first choice was 3-Methyl-histidine, an amino-acid which comprises a relatively large proportion of the myofibrillar proteins of muscle cells. However, the cost of this molecule in radiolabelled form, was prohibitive. As an alternative, carnitine (γ-amino-β-hydroxybutyric acid 3-methyl betaine) looked to be a promising candidate. This thesis describes how my interests in muscle cell physiology and immunology combined in a study of the immunopathogenesis of inflammatory muscle disease.

SECTION II

MATERIALS AND METHODS

II MATERIALS AND METHODS

CHAPTER 1

II.1 PATIENTS

II.1.1 Source and selection of patients

(i) Adults. The adult patients studied were attending the Rheumatology out-patient clinic at Hammersmith Hospital (HH) under the care of Dr GRV Hughes, or the University College Hospital (UCH) Rheumatology out-patient department under the care of Dr M L Snaith. The clinical diagnosis and assessments of individual patients were kindly provided at the completion of the lymphocyte studies described in this thesis by Dr M Walport at HH and Dr D Isenberg at UCH. Relevent clinical details, drug therapy and laboratory results are summarised in Section IV.

(ii) Children. Parental approval was obtained on all occasions when blood was taken from children in these studies. All the children were attending the Muscle Clinic at Hammmersmith Hospital under the care of Prof V Dubowitz, in the Department of Paediatrics and Neonatal Medicine.

The case histories of three children studied over a two year period (Chapter IV.4) were written with the help of Dr J Z Heckmatt, Senior Research Registrar, Dept of Child Health at Hammersmith Hospital.

(iii) Controls. Control subjects were all members of staff at Hammersmith Hospital.

II.1.2 Collection and preparation of blood samples

(i) Collection of blood samples. For the lymphocyte studies described in Section III, 20-60 ml of venous blood was taken from patient or control subjects and defibrinated mechanically with sterile orange sticks. This method of defibrination was used in preference to that of the use of heparin as an anti-coagulant, because it enabled collection of serum from each patient without the need to take extra blood for a clotted sample.

(ii) Separation of peripheral blood mononuclear cells: Following defibrination, blood samples were centrifuged on an MSE Minor bench centrifuge at 200g for 10 min to pellet the cells. Serum was removed and stored in 500 µl aliquots at -20°C. An equal volume of calcium and magnesium-free Hanks' Balanced Salt Solution (HBSS-Grand Island Biological Company) was added to the cell pellet. Peripheral blood mononuclear (PBM) cells were obtained by centrifugation over a discontinuous density gradient of Ficoll-Hypaque (Winthrop Laboratories) after the method of Böyum (1968). Twelve ml aliquots of the cell suspension were gently layered over 10 ml of Ficoll-Hypaque previously adjusted to a specific gravity of 1.078, and centrifuged at 400g for 20 min. Mononuclear cells were removed from the Ficoll-Hypaque/HBSS interface and washed twice in RPMI-1640 tissue culture medium (Flow Laboratories) supplemented with 2 mM L-Glutamine, 20 µg/ml gentamicin and buffered with 0.25% sodium bicarbonate and 10 mM HEPES.

This basic culture medium with supplements will be referred to as maintainance medium and abbreviated as MM.

The PBM cells were finally resuspended at a concentration of 1 x 10^6 ml in MM supplemented with 5% Fetal Calf Serum (FCS) (Flow Labs.) and stored at 4 °C overnight before being used in cytotoxicity experiments. This procedure was strictly adhered to in order to standardise the culture conditions under which individual experiments were performed, and was necessary because of the widely varying times of day when blood samples were made available.

The following morning, the PBM cells were allowed to warm to room temperature, centrifuged for 5 min at 200g and resuspended in MM containing 10% FCS.

(iii) Removal of adherent cells from PBM populations: For removal of plastic-adherent cells, which are primarily monocytes, the concentration of PBM cells was adjusted to 2 x 10^6/ml and 5ml aliquots of these suspensions added to 25cm^2 plastic tissue culture flasks (Falcon) and incubated for 45 min at 37°C in a humidified atmosphere of 5% CO_2 in air. Following incubation, the flasks were gently agitated, the non-adherent cells removed and washed once in MM. These plastic adherent cell depleted PBM populations, most of which had the morphological appearance of lymphocytes, will be referred to as peripheral blood lymphocytes (PBL). Contamination by monocytes in these populations was monitored by staining for non-specific esterase-positive cells. If there were greater than 2% non-specific esterase-positive staining cells in PBL samples, the plastic adherent cell

depletion step was repeated. However, this was seldom necessary.

(iv) Removal of Fc-receptor bearing lymphocytes from PBL: A solid phase immunosorbent technique modified after that of Kedar, de Landazuri and Bonavida, (1974) was used to remove lymphocytes which bear receptors for the Fc portion of IgG molecules ($Fc_{\gamma}R$ lymphocytes). Ox red blood cell (OE) monolayers were prepared in poly-L-lysine (PLL) (Sigma Chemical Company) coated 25 cm^2 tissue culture flasks. The flasks were incubated at room temperature with 2.5 ml of 100 µg/ml of PLL of molecular weight 400,000 which was prepared in phosphate buffered saline (PBS) at pH 7.3. After 45 min, the flasks were rinsed 5 times with 5 ml aliquots of PBS and 2.5 ml of 1.5% (volume/volume) suspension of fresh (up to 8 days old) ox erythrocytes which had been previously washed 4 times in PBS, were added.

The flasks were left at room temperature for 45 min and then washed repeatedly with PBS until a confluent, homogeneous monolayer of OE was obtained. The monolayers were covered with 3 ml of PBS and stored at 4° C until use within 3 days of preparation.

Before fractionation of lymphoid cells, the PBS was removed from the plates and 2.5 ml of the IgG fraction of rabbit anti-ox erythrocyte serum (Sigma Chemical Company) previously adjusted to the maximum non-agglutinating dilution in PBS, was added. Control OE monolayers were treated with a 1 in 5 dilution of normal rabbit serum, which had been absorbed twice with 1:1 volumes of packed ox erythrocytes, were included for use in corresponding experiments. After 45 min incubation at 37 °C, the serum was removed and the monolayers washed gently four

times with PBS. After the final wash, 3 ml of MM supplemented with 10% FCS was added to each flask which were left at room temperature for 10 min. This medium was then decanted and 2 mls of PBL at a concentration of 10 cells per ml added to each flask. Following 15 min incubation at 37°C in an atmosphere of 5% CO_2 in air, the flasks were centrifuged at 100g for 5 min at 24°C. This was done by taping the flasks to microtitre plate carriers on the L 32 swing out rotor of a Mistral 6L centrifuge. After centrifugation, the flasks were gently agitated and the supernatant removed. The monolayers were washed once with 3 ml of MM containing 10% FCS and, after gentle agitation, the medium removed. The two fractions containing the non-adherent cells were combined, washed once with MM+10% FCS and the concentration adjusted to that required for use in further experiments. No loss in function was observed in PBL populations fractionated in this way.

CHAPTER 2

II.2 CELL CULTURES

II.2.1 Fetal tissue culture

(i) Ethical approval. Following approval by the Research Ethics Committee, human fetal tissue, suitable for culture was made available for this study, by the Institute of Obstetrics and Gynaecology at Hammersmith Hospital.

(ii) Selection of tissue. Fetuses aborted by the suction termination method at 9-12 weeks gestation were collected within one hour of the operation. The age of the fetus was determined by fetal foot length, by a member of the Department of Child Health. Fetal material from women undergoing abortion because of a probable Rubella virus infection in the early stages of pregnancy, was not used.

(iii) Tissue culture.

(a) Skeletal muscle. Skeletal muscle was removed from the limbs of the fetus by dissection and placed in 100 mm diameter glass petri dishes containing 2-3 ml of Medium 199 (Flow Laboratories) buffered with 0.35% sodium bicarbonate, gentamicin at a concentration of 50 µg/ml and L-glutamine at 2 mM/ml. This culture medium will be referred to as CM. The muscle tissue was finely chopped with a sterile scalpel and then transferred to 30 ml plastic universal containers containing 10 ml of a mixture of 0.025% trypsin (Flow Laboratories) and 0.5 mM

disodium ethylene diamine tetra-acetic acid (EDTA) in PBS (trypsin/EDTA). These were then placed on an angled rotary mixer, set at 30 revolutions per minute in a 37°C hot room for 30 min.

Following incubation, 2 ml of heat inactivated fetal calf serum was added to each in order to stop the action of trypsin. The tubes were then centrifuged at 200 g for 10 min. The pellet was resuspended in 10 ml of CM and then left to stand at room temperature for 5 min in order to allow the largest clumps of partially-digested material to sediment. The supernatant, containing smaller clumps of cells and released single cells, was transferred to fresh universal containers and centrifuged at 200g for 10 min.

The pellet was resuspended in 10 ml of CM and filtered through a double layer of sterile gauze cloth in order to remove the smaller clumps of undigested fibrous tissue. The cells in the filtered suspension were then counted and following another washing step, the concentration was adjusted to 5 x 10^5 cells per ml in CM supplemented with 10% FCS ready for pre-plating.

The process of pre-plating suspensions of cells released by trypsin digestion of skeletal muscle was originally suggested by Kaighn, (1966). The method, modified by Yaffe (1968) is designed to remove some of the fibroblasts from the mixed cell suspensions of myoblasts and fibroblasts which are present in dissociated skeletal muscle tissue. The cells identified morphologically as fibroblasts settle and attach more rapidly than myoblasts. Four ml aliquots of the filtered cell suspension were added to 25 cm^2 plastic tissue culture flasks

(Falcon Plastics Ltd) and incubated for 10 min at 37°C. The non-adherent cells were removed, washed once in CM and then cultured in 25 cm^2 tissue culture flasks in 5 ml of CM supplemented with 10% FCS at a concentration of 1 x 10^5 cells per ml.

The flasks were incubated at 37°C in an atmosphere of 5% CO_2 in air, cultures being examined daily. When the cultured cells had formed a confluent monolayer, the culture medium was removed, 1 ml of trypsin/EDTA added and the flasks incubated at 37°C for 5 min in order to detach the cells from the plastic. Culture medium was then added and, following one washing step, the cells were resuspended in CM, supplemented with 5% FCS and subcultured into two 25 cm^2 flasks for each original flask of confluent cells. When the cultures were again close to confluence, usually within 48 to 72 hours, the cells were again detached by trypsin/EDTA, washed once in CM and finally dispersed in 96 - well flat-bottomed tissue culture plates (Falcon Plastics Ltd.) at a concentration of 1-2 x 10^5 cells per well in a volume of 200 µl CM, supplemented with 2% FCS. The 36 wells on the outside edges of the 96 well microtitre plates were not used for cell culture, because it was found that cells seeded into those wells did not thrive. Culture medium alone (200 µl vol) was added to these outside wells.

For radioautographic studies, 1 x 10^6 cells were finally dispersed into 35 mm diameter plastic Petri dishes (Falcon Plastics Ltd) in 3 ml of CM, supplemented with 5% FCS. Culture was continued for between 7 and 10 days at 37 °C in a humidified atmosphere of 5% CO_2 in air, half of the culture medium being removed every 2-3 days and replaced with CM and a reduced concentration of FCS (1-2%) which allows myoblast

fusion to proceed optimally. Similarly, 100 l of medium was removed from each microplate culture every 2-3 days and replaced with fresh CM, + 2% FCS.

The maturity of the microplate and Petri dish muscle cultures was assessed by examining each culture by inverted phase microscopy at a magnification of 200 x (Fig II.2.1). Only those cultures containing a confluent monolayer of healthy cells and greater than 6 multinucleate (usually 2 to 8 nuclei per cell) myotubes per field were used in subsequent experiments.

(b) Skin fibroblast cultures. Fibroblast cultures were established from pieces of skin dissected from the back of the fetus. Cells were released from the finely chopped pieces of skin by trypsin/EDTA digestion, as in the method used for fetal muscle culture. After filtration to remove clumps of undigested material, the concentration of cells was adjusted to 2 x 10^5 per ml of CM+ 10% FCS and then added in 5 ml aliquots to 25 cm plastic tissue culture flasks.

These primary cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air until confluent. They were then subcultured by the trypsin/EDTA method, being split 1 to 3 into 25 cm^2 tissue culture flasks.

On the day preceeding experiments of ^{3}H-Carnitine uptake and loss (Section III), confluent monolayers of skin fibroblasts were detached by trypsin/EDTA, washed once in CM and the concentration adjusted to 5 x 10^5 cells per ml of CM, supplemented with 5% FCS. One hundred

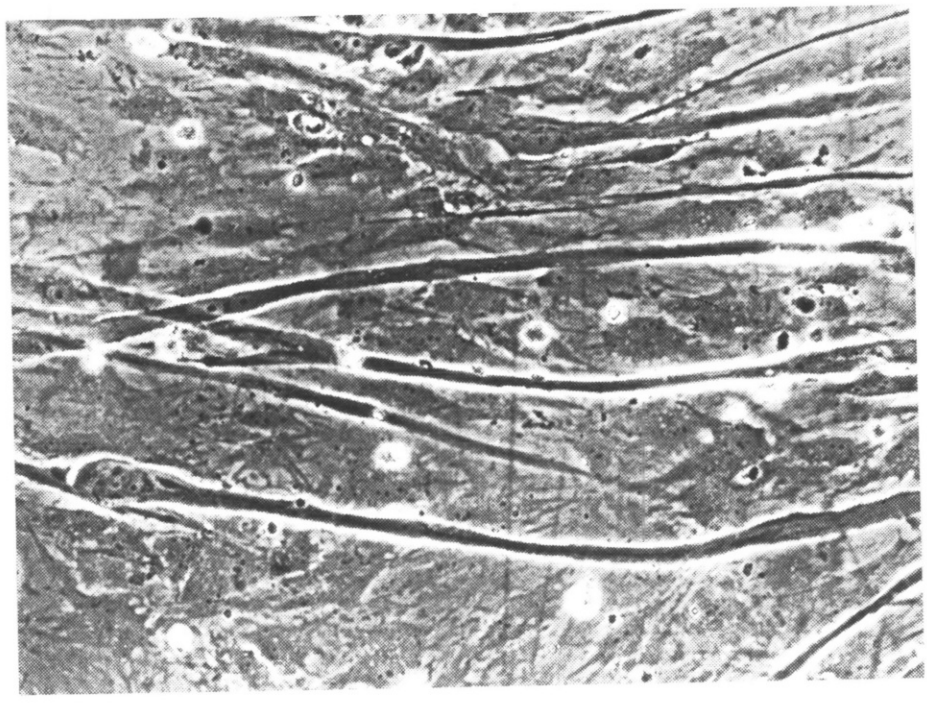


Figure II.2.1

Phase-contrast photomicrograph of human fetal muscle, disaggregated and grown in culture for 13 days, showing the large myotubes (pre-muscle cells) growing on and among fibroblasts. Magnification x 300.

microlitres of this cell suspension were added to each well of flat-bottomed tissue culture plates. As with the microplate cultures of human fetal skeletal muscle, the 36 wells on the perimeter of the plate were filled with CM, not cells. This concentration of skin fibroblasts (5×10^4 /well) was sufficient to ensure a confluent monolayer of cells in each well by the following day. In experiments where human fetal skin fibroblasts (HFF) were used as targets for lymphocyte effector populations, the cells were sub-cultured twice in 25 cm^2 flasks before use.

II.2.2 Continuous cell lines

(i) K562 cell line

(a) Source and characteristics. The K562 cell line was obtained from Dr K Welsh, Tissue Typing Laboratories, Guy's Hospital, London.

This cell line was first established in vitro and described by Lozzio and Lozzio in 1975. It was derived from cells present in the pleural effusion of a patient with chronic myelocytic leukeumia, who was in blast crisis. The K562 cell line possesses some of the phenotypic surface markers of human T-cells, but does not express products of the major histocompatability (MHC) locus in detectable amounts (Andersson, Nilsson and Gahnberg, 1979).

(b) Maintenance of K562 cell line. In vitro, K562 cells grow as suspension cultures. In our laboratory, they were maintained as stock cultures in 75 cm^2 plastic tissue culture flasks (Falcon Plastics Ltd),

standing upright in a 5% CO_2 in air atmosphere at 37°C. The cells were kept at a concentration of between 5 and 10 x 10^3 cells per ml in 20-30 mls per flask of RPMI-1640 medium (Flow Laboratories) containing sodium bicarbonate (2.0 mg/ml), 0.4 mg/ml L-Glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin, and 5% fetal calf serum (known as growth medium - GM). When the concentration of cells exceeded 10 x 10^4/ml, half of the cell suspension was discarded and replaced with an equal volume of fresh GM. The cells were screened for mycoplasma contamination every month by the Department of Microbiology at Hammersmith Hospital. If the cells showed positive fluorescent staining for mycoplasma, all cultures were discarded and known mycoplasma free, K562 cells restored from frozen stocks.

(ii) Girardi heart cells

a) Source and characteristics The Girardi heart cell line was obtained from Flow Laboratories (American Type Culture Collection CCL 27). This line was derived from the right atrial appendage of a child who died from non- cardiac related causes (Girardi, 1957) and grows as a monolayer in culture. The cells are epithelial-like in morphology and contain myosin ATP-ase activity. Antisera raised to Girardi heart cells in rabbits, reacted only with the myocardial, and not with the fibroblastic components of human fetal heart monolayer cultures (Cambridge and Davis, unpublished observations).

b) Maintainance of Girardi heart cells. The Girardi heart cells were maintained in GM in 75 cm^2 tissue culture flasks and passaged twice weekly, or whenever monolayers were confluent.

c) Uptake of ^{3}H-Carnitine by Girardi heart cells. On the day preceding experiments to measure ^{3}H-Carnitine uptake by Girardi heart cells, the cells were detached from the plastic flasks by incubation with trypsin/EDTA, washed twice in MM and cultured in flat bottomed microtitre plates at a concentration of 5×10^{4} cells per well in 100 µl GM. This concentration of cells was sufficient to give a confluent monolayer of cells for use in experiments the following day.

CHAPTER 3

II.3 NATURAL KILLER CELL ASSAYS

II.3.1 Labelling of cells with ^{51}-Sodium Chromate for Natural Killer cell assays

(i) K562. Between 5 and 10 x 10^6 K562 cells were washed once in GM and resuspended in 0.5 ml of culture medium without fetal calf serum. To these cells were added 100 µCi of 51-sodium chromate (^{51}Cr) (Amersham Radiochemicals) which had a specific activity of between 250 and 500 µCi per µg of sodium chromate. They were then incubated for 45 min at 37°C in an atmosphere of 5% CO_2 in air, with occasional gentle mixing. Following incubation, the labelled cells were washed twice in MM and reincubated in a 10 ml volume of MM, supplemented with 10% FCS for a further 30 min. The number of viable cells per ml was then calculated, and following one further washing step, the concentration was adjusted to 10 x 10^4 viable cells per ml in MM + 10% FCS. The labelled cells were then dispensed in 100 µl volumes into 96 well round-bottomed microtitre plates (Nunc), ready for use in conventional short term (4 hour) natural killer cell assays. (Roder et al., 1978).

(ii) Human fetal skin fibroblasts. Human fetal skin fibroblasts (HFF) were established as described in II.2.1. They were used as targets for human PBL effector cell populations after their second in vitro passage. Confluent monolayers of HFF were detached from plastic flasks in which they were cultured, by trypsin/EDTA incubation, washed twice in MM and resuspended in 0.5 ml of serum free medium. One hundred

microcuries (100 µCi) of ^{51}Cr specific activity 250-500 µCi/mg sodium chromate, were added to the cells which were then incubated for 1 hour at 37°C, with gentle mixing every 15 min. Following incubation, the cells were washed thrice in MM and their concentration adjusted to 10 x 10^4 cells per ml of MM supplemented with 10% FCS. One hundred microlitres of this cell suspension were added to the appropriate number of wells in flat-bottomed 96 well microtitre plates (Falcon Plastics Ltd). The plates were incubated for one hour at 37 °C in a humidified atmosphere of CO_2 in air, by which time all the cells had adhered to and spread out on the bottom of each well in an even monolayer. The supernatant was then gently removed from each culture and labelled HFF monolayers each washed with two successive 200 µl volumes of MM supplemented with 5% FCS, in order to remove any ^{51}Cr which was released during adherance of the cells to the bottom of the microtitre plate wells. One hundred microlitres (100 µl) of MM supplemented with 10% FCS was then added to all the HFF microcultures. These ^{51}Cr labelled monolayers were then used immediately in assays of lymphocyte-mediated cytotoxity.

II.3.2 Natural Killer cell assays.

Human peripheral blood lymphocyte effector populations, prepared as prevously described (Chapter II.1), were adjusted to 10, 5 and 2.5 x 10^6 cells per ml of MM + 10% FCS (unless otherwise indicated) and added in 100 µl aliquots to triplicate wells containing either ^{51}Cr-labelled K562 cells or human fetal skin fibroblasts. To those wells containing target cells alone, which were to serve as controls for the spontaneous release of ^{51}Cr from labelled cells during the course of the assay,

100 µl of MM + 10% FCS was added.

In addition, for measurement of the maximum ^{51}Cr release possible from the labelled cells, 100 µl of 6% sodium dodecyl sulphate (SDS) was added to triplicate wells containing either K562 cells or skin fibroblasts. The plates were incubated at 37°C for 4 hours, when K562 cells were the target cells, and for 7 hours, when HFF were the targets, in a humidified 5% CO_2 in air atmosphere. Following incubation, the microtitre plates were centrifuged at 100 g for 5 min in a Mistral 6L centrifuge fitted with an LR 32 swing out rotor. One hundred microlitre (100 µl) aliquots of supernatant was removed from each well, and radioactivity per sample measured for 2 min on an LKB gamma counter. The average counts per minute (cpm) of triplicate samples were used to calculate percentage cytotoxity for effector lymphocyte populations by the following formula.

$$\% \text{ cytotoxicity} = \frac{(\text{cpm}^{\text{test release}}) - (\text{cpm}^{\text{spontaneous release}})}{(\text{cpm}^{\text{maximal release}}) - (\text{cpm}^{\text{spontaneous release}})} \times 100$$

CHAPTER 4

II.4 LYMPHOCYTE-MEDIATED MYOTOXICITY ASSAYS

II.4.1 Introduction:

The method used for measuring lymphocyte mediated cytotoxicity against human fetal muscle (HFM) cultures was developed following studies of the physiology of ^{3}H-Carnitine uptake and loss from such cultures, as described in Section III. These experiments showed that ^{3}H-Carnitine was specifically taken up by the muscle cells, and not by fibroblasts which were also present in cultures derived from human fetal muscle. The assay used for these studies is based on the reduction in retention of ^{3}H-Carnitine by pre-labelled cultures of human fetal muscle, following incubation with peripheral blood lymphocyte populations.

II.4.2 Labelling cultures of human fetal muscle with ^{3}H-Carnitine

Mature healthy cultures of human fetal muscle (HFM) were prepared in 96-well microtitre plates as described in II.2. On the day preceding an experiment, the culture medium was removed from each well. Tritium-labelled carnitine (^{3}H-C) obtained from Amersham Radiochemicals Ltd., and of specific activity 750 to 1,700 mCi/mMole, was diluted in RPMI 1640 medium (Flow Laboratories Ltd.) with MM, to give a final molarity of 0.59 to 1.33 µmol/l. One hundred microlitres of this solution, containing 0.1 Ci of tritium, was added to each well. The cultures were incubated overnight (12-14 hours) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Following incubation, each well was

washed three times with MM, then 50 µl of CM added to all wells. Peripheral blood lymphocyte (PBL) populations prepared as previously described (CH II.1) were adjusted to 10, 5, 2.5 or 1.25 x 10^6 per ml in CM (unless otherwise indicated) and 100 µl of each suspension added to each of 4 replicate wells, each containing a muscle culture. Control wells, 4 of which received 100 l of CM alone, and 4, 100 µl of 6% sodium dodecyl sulphate (SDS), were also included on each plate. Incubation at 37°C, in an atmosphere of 5% CO_2 in air, was continued for 18 hours in preliminary experiments (Chapter IV.2) and for 7 hours in all other experiments (Chapters IV 3-7).

II.4.3 Harvesting of myotoxicity assays

Following incubation, wells which had received either lymphocytes, or CM alone, were washed three times with MM, 100 µl of 6% SDS were added to all wells and the plate allowed to stand overnight at room temperature. The contents of each well were transferred to scintillation vials and each well washed twice with 200 µl volumes of water, the washings being transferred also to appropriate scintillation vials. To each vial was added 2 ml of Unisolve 100 scintillation fluid (Koch-Lite Ltd). They were then mixed on a vortex mixer and allowed to stand overnight at 4 °C. Emmission of β-particles was measured photometrically on an LKB Rack-Beta scintillation counter.

II.4.4 Radioautography; labelling techniques

Monolayer cultures of human fetal muscle grown in 35 mm-diameter petri dishes, were incubated with one ml of CM containing 2 µCi of tritium-

labelled carnitine (specific activity 1.7 Ci/mMole) for 14 hours at 37°C in 5% of CO_2/air. The CM was then removed and the dishes washed 3 times with MM before being snap-frozen by floating the dishes on liquid nitrogen. The frozen monolayers were dried in a freeze drier (Chemlab Industries) to a vapour pressure of 50 m Torr and kept under vaccuum at 4 °C in a glass dessicator. At radioautography, several 1 cm pieces were cut from the bottom of each dish with a hot scalpel and placed in turn on a 1 cm diameter stainless-steel post in a dark room. A thin film of high-plasticiser K-photographic emulsion (Ilford Film Company) was taken upon a 2 cm diameter platinum wire loop mounted on a glass rod, and placed over each 1-cm piece. The films were dried rapidly in air and exposed in light proof boxes for 2-3 weeks. The preparations were developed in D 19 developer (Kodak), stopped in 3% acetic acid and fixed in a 1.3 dilution of Amfix (Kodak), before staining the cell monolayers with haematoxylin.

SECTION III

THE PHYSIOLOGY OF CARNITINE UPTAKE BY HUMAN FETAL MUSCLE CULTURES

III PHYSIOLOGY OF CARNITINE

CHAPTER 1

III.1 INTRODUCTION

III.1.1 Importance of carnitine to fatty acid metabolism

Carnitine (β-hydroxy γ-trimethylammonium butyrate) was first described as "an extractable substance from muscle" by Gulewitsch and Krimberg in 1905 and its importance in fatty acid metabolism was established by Fritz and Marquis (1965).

The function of carnitine in the eukaryote cell, is to assist the transport of long chain fatty acids (greater than 12 carbon atoms in the acyl chain) across the mitochondrial membrane (Greville and Tubbs, 1968). These fatty acids are then available for β-oxidation (resulting in the formation of acetyl Co-A which then enters the Krebs cycle) inside the mitochondria. Thus, carnitine is of major importance to the metabolism of those tissues which rely on fatty acid oxidation as a principal source of energy, for example the heart and skeletal muscle in mammals.

Carnitine facilitates the transport of long chain fatty acids across the inner mitochondrial membrane, Carnitine palmityl transferase, which catalyses the reversible reaction of carnitine with long chain fatty acyl groups is required for this process. Carnitine deficiency (Engel and Angeline 1973) and carnitine palmityl transferase deficiency

(Karpati et al., 1975) have been identified as principal causes of lipid storage myopathy in man (Boudin et al., 1976). Heriditary carnitine deficiency has also been described by Vandyke et al (1975). These defects are characterised by progressive weakness and increased content of lipid vacuoles in type I muscle fibres in carnitine deficiency, and by periodic muscle cramps and pigmenturia in the deficiency of carnitine palmytyl transferase.

III.1.2 Biogenesis

Results of investigations in rats indicated that carnitine is derived from lysine and most, if not all, synthesis occurs in the liver (Lindstedt, 1967). The carnitine synthesized in the liver is released into the bloodstream and transported to other tissues. Although different organs are perfused with blood having the same carnitine concentration, the carnitine concentration varies from tissue to tissue. It is especially high in the heart and skeletal muscle (Christianson and Bremer, 1976).

Carnitine biogenesis was first studied in the mould Neurospora crassa (Horne et al., 1971). Carnitine is synthesized from lysine and methionine precursors. It was subsequently shown that carnitine was synthesized by the same pathway in the rat (Tanphaichitr, Horne and Broquist, 1971). However, while most tissues can carry out the early steps in carnitine biosynthesis, the final reaction in the pathway, hydroxylation of -butyrobetaine, occurs primarily in the liver and not at all in the heart or skeletal muscle. As previously stated, these tissues have the highest intracellular concentration of carnitine,

indicating that carnitine is either trapped within the muscle or that a specific concentrating mechanism is present in these tissues.

III.1.3 Selective uptake of carnitine

In the eukaryote cell, selective transport of carnitine across the plasma membrane of liver and heart cells in culture has been described. Bohmer, Eiklid and Jonsen, (1977), studied the kinetics of carnitine transport by the human heart cell line (Girardi CCL 27) and the mouse fibroblast cell line (L929) *in vitro*. Uptake by Girardi heart cells was more than three times greater than that by fibroblasts after four hours of incubation with tritium labelled carnitine. The addition of 2-4 dinitrophenol, an agent known to uncouple oxidative phosphorylation, or denaturation of the heart cells by heat or trichloro-acetic acid, effectively reduced the carnitine uptake. Thus, the uptake of carnitine seemed to be dependent upon an energy-requiring transport mechanism. Sequestration of carnitine, or binding to long chain fatty acid residues, did not account for the ability to take up carnitine against a concentration gradient. In analogous experiements, the selective uptake of carnitine was also demonstrated in studies of whole isolated muscles from rats (Buse et al., 1972; Willner, Ginsburg and Dimauro, 1978; Rebouche, 1977). Transport of carnitine into the whole muscle was also blocked by respiratory inhibitors such as 2-4 dinitrophenol, sodium azide and anaerobiosis. In addition, involvement of the plasma membrane sodium and potassium triphosphatases in saturable carnitine uptake was suggested by inhibition of the uptake process by ouabain, and by the deprivation of sodium or potassium ions from the incubation solution. This carnitine-concentrating mechanism

was evident at the physiological concentration of carnitine in the plasma of the rat and was concluded to be of importance for normal carnitine uptake by skeletal muscle *in vivo*.

III.1.4 Summary of the role of carnitine in mammalian metabolism

The vital role of lipid metabolism in the generation of energy is well established, as is the ability of skeletal and cardiac muscle to exist on carbohydrate free lipid energy stores. Carnitine is essential, as it acts as a 'carrier' molecule for long chain fatty acids across the inner mitochondrial membrane, to make them available for β-oxidation within the mitochondria. Carnitine cannot be synthesized by muscle, however its concentration within muscle is higher than in other tissues or in plasma. Kinetic studies of carnitine uptake by cultured human heart cells and whole isolated rat muscle have shown that energy-requiring membrane- associated selective uptake mechanisms probably operate for carnitine *in vivo*. Defects in carnitine biosynthesis or of carnitine uptake by muscle from the blood plasma is one of the most common causes of lipid storage myopathy in humans.

III.1.5 Carnitine: a potential in vitro marker for muscle cells in cultures of human fetal muscle.

When skeletal muscle tissue is disaggregated and cultured *in vitro*, a population of two cell types develops; myoblasts and fibroblasts. The myoblasts, or pre-muscle cells, divide several times before fusing and developing into large multinucleate myotubes, which lie among the rapidly dividing fibroblasts. Microscopically, it is possible easily

to distinguish the myotubes from the background of fibroblasts. However, when such cultures become damaged, it is difficult to determine which cell type has been most affected. The purpose of experiments described in this section, was to determine whether tritium labelled carnitine was a selective marker for muscle cells in the mixed cell populations present in cultures derived from human fetal muscle tissue. As described previously in this chapter, the kinetics of tritium-labelled carnitine uptake by pure cultures of human heart cells and of fibroblasts have shown that a selective active transport mechanism for carnitine was present in the heart cells and not the fibroblasts cells. A study of the kinetics of carnitine uptake by cultures of human fetal muscle cultures and human fetal skin fibroblasts was undertaken to determine whether a similar concentrating mechanism for carnitine exists in cultured human fetal muscle cells.

CHAPTER 2

III.2 ^{3}H-CARNITINE UPTAKE BY HUMAN FETAL MUSCLE: PRELIMINARY STUDIES

III.2.1. Introduction

The experiments in this chapter were designed to see whether cultures of human fetal muscle (HFM) could selectively remove carnitine from the culture medium in which they were incubated. Carnitine labelled with tritium (^{3}H-C) was the only source of carnitine available to the cultures.

III.2.2. Disappearance of carnitine from HFM supernatants

(i) Experimental design. Cultures of human fetal muscle (HFM) were established as described in II.1. The growth medium was removed from each culture of HFM grown in 96 well microtitre plates and replaced with 100 µl of RPMI 1640 medium (FLow Laboratories Ltd.) supplemented with 2 mg/ml sodium bicarbonate and 2 mM L-glutamine containing either 5, 0.5 or 0.05 µCi ^{3}H-Carnitine (specific activity 900 mCi/mMole) per ml, and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. At intervals thereafter, supernatants from replicates of 4 wells were removed and transferred to scintillation counting vials. One millilitre of Unisolve 100 liquid scintillation fluid (Koch-Lite Laboratories) was added to each vial and mixed on a vortex mixer. The vials were left at 4°C overnight and β-emission due to tritium measured photometrically on an LKB Rack-Beta scintillation counter. The mean counts per minute (CPM) of four replicate supernatants were calculated

and are represented in Table III.2.2. These values were also expressed as a percentage of the total carnitine added initially.

(ii) Results. In this experiment, tritium labelled carnitine (^{3}H-C) was added to cultures of HFM and its change in concentration in the supernatant followed with time. At all three concentrations of ^{3}H-C added, approximately 10% of the labelled compound was lost from the supernatant within 4 hours of incubation (Table III.2.2). After twenty four hours incubation, only 12-15% (approximately) had disappeared from the supernatant.

III.2.3. The distribution of ^{3}H-C in (HFM) supernatants and monolayers

(i) Experimental design. In this experiment, the partitioning of ^{3}H-C between monolayer cultures of HFM and culture supernatants was determined. The experiment was performed as in III.2.2.(i), however, after removal of the supernatant at invervals of incubation, the monlayers were dissolved in 6% SDS and the contents transferred to scintillation vials. Each well was then washed with two 200 µl volumes of water, the washings pooled and transferred to the appropriate vials for estimation of tritium present. After 1.5 ml of liquid scintillation fluid had been added, the tubes mixed and left at 4°C overnight, β-emission due to ^{3}H-C was measured. The mean percentage of ^{3}H-C associated with replicates of four HFM monolayers or supernatants was calculated and expressed as a percentage of the total ^{3}H-C (cells + supernatant) recovered from the corresponding wells. The results are expressed graphically in Fig III.2.3 for each ^{3}H-C concentration added to 4 replicate wells.

µCi ^{3}H-Carnitine added/ well	Time of incubation (hours)					
	0	2	4	6	8	24
0.5	315,724	313,319	290,006	289,228	280,372	280,532
	(100)	(99.23)	(91.85)	(91.61)	(88.80)	(88.85)
0.05	35,034	33,269	30,855	31,057	31,160	29,287
	(100)	(94.96)	(88.07)	(88.65)	(88.94)	(83.36)
0.005	3,456	3,360	3,318	3,449	3,096	2,972
	(100)	(97.22)	(96.01)	(94.01)	(89.58)	(85.99)

Table III.2.2

The mean counts per minute of ^{3}H-Carnitine present in supernatants from 4 replicate wells of human fetal muscle at increasing time intervals, following incubation with either 0.5, 0.05 or 0.005 µCi H-Carnitine per well. (specific acitivity 900 mCi/mMole carnitine). The ^{3}H-Carnitine present in supernatants is also present as a percentage of ^{3}H-Carnitine added at the initiation of the experiment, in parentheses.

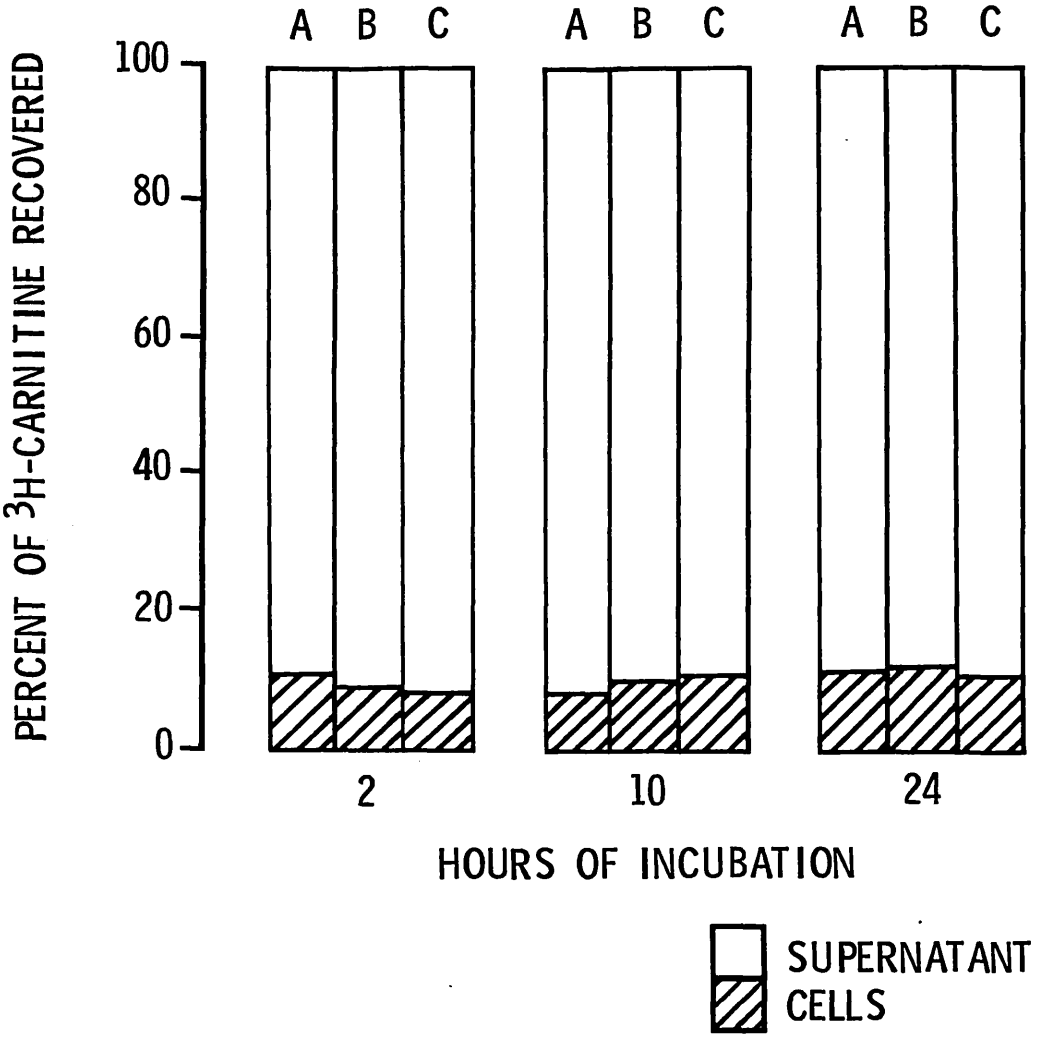


Figure III.2.3

The partitioning of ^{3}H-Carnitine into cellular or supernatant fractions of human fetal muscle cultures after 2, 10 and 24 hours incubation with either 0.5 µCi (A columns), 0.05 µCi (B columns) or 0.005 µCi (C columns) H-Carnitine per well (specific activity 900 mCi/mMole carnitine). Open bars represent the percentage of added radiolabel recovered from supernatants and cross-hatched bars, that associated with cellular fractions.

(ii) Results. Fig III.2.3 shows that the disappearance of ^{3}H-C from supernatants was synchronous with its association with the cell monolayers. Over the range of ^{3}H-C concentrations added to these cultures, approximately 10% of the label was found to be associated with the cellular fraction within two hours of incubation. This had not significantly increased after 10 or 24 hours incubation.

III.2.4. Discussion

Carnitine is a hydrophobic molecule and would therefore tend to associate with polar molecules such as those lipids present in cell membranes. The rapid association of ^{3}H-C with the monolayer cultures of muscle within 2 hours of incubation and no increase after 10 or 24 hours incubation (Fig III.2.3) could reflect non-specific attraction between this molecule and lipids present in the monolayer cell membrane. Even in the presence of a membrane associated transport system, any specific uptake or diffusion of ^{3}H-C into the cultures might be masked to a certain extent, provided that there was a large difference in the first order kinetics of passive adsorbtion compared with selective uptake. This would then account for the lack of difference between two and ten hour values and only slightly more ^{3}H-C disappearing from the supernatant after 24 hours incubation.

CHAPTER 3

III.3 WASHING EXPERIMENTS

III.3.1 Introduction

It was possible that a proportion of the tritium labelled carnitine added to the supernatant medium of cultures of human fetal muscle rapidly associated non-specifically with the surface membranes of the cells because of its hydrophobic nature. This could potentially mask any selective uptake of ^{3}H-C by the cultures. The following experiments were undertaken to determine the proportion of ^{3}H-C remaining following successive washes of the monolayers with culture medium after incubation with medium containing ^{3}H-C.

III.3.2 Washing experiments

(i) Experimental design. Replicates of four wells, each containing differentiated human fetal muscle monolayers grown in 96 well microtitre plates were incubated with 100 µl of culture medium containing either 10 or 1 µCi ^{3}H-C (specific activity 750 mCi/mMole) per ml for 8 and 20 hours, in an atmosphere of 5% CO_2 in air at 37°C. At the end of the incubation periods, the supernatants were removed and the monolayers washed gently with 200 µl of culture medium. After each successive washing step, the replicate cultures were dissolved in 6% SDS and harvested for estimation of β-emission due to tritium as in III.2.

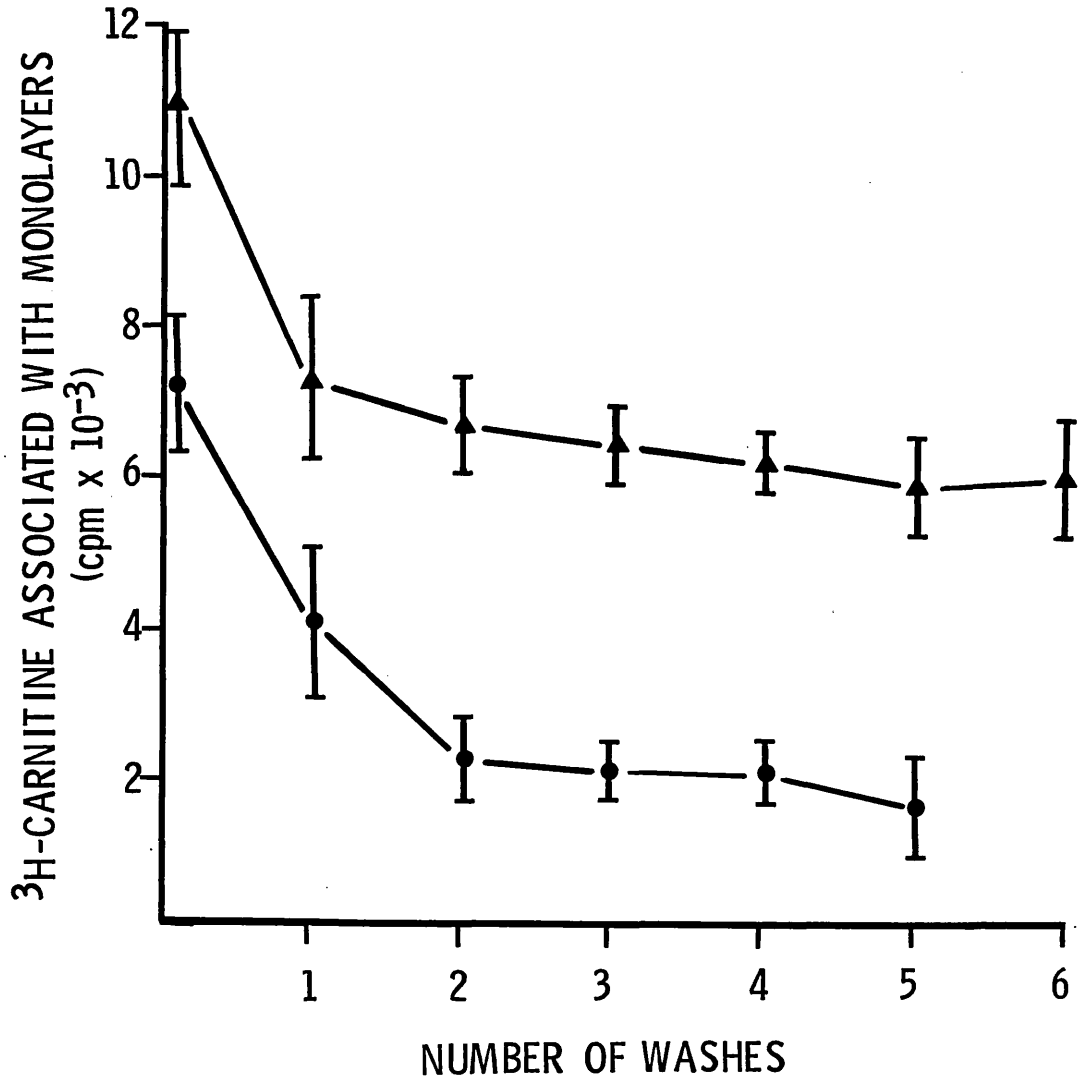


Figure III.3.2.i

The mean counts per minute (cpm) obtained after solubilising human fetal muscle monolayers, following 8 hours incubation with either 0.1 µCi (▲) or 0.01 µCi (●) ^{3}H-Carnitine (specific activity 900 mCi/mMole carnitine) and successive washes of the monolayers with 200 µl culture medium. Horizontal bars represent one standard deviation from the mean values given by 4 replicate wells for each determination.

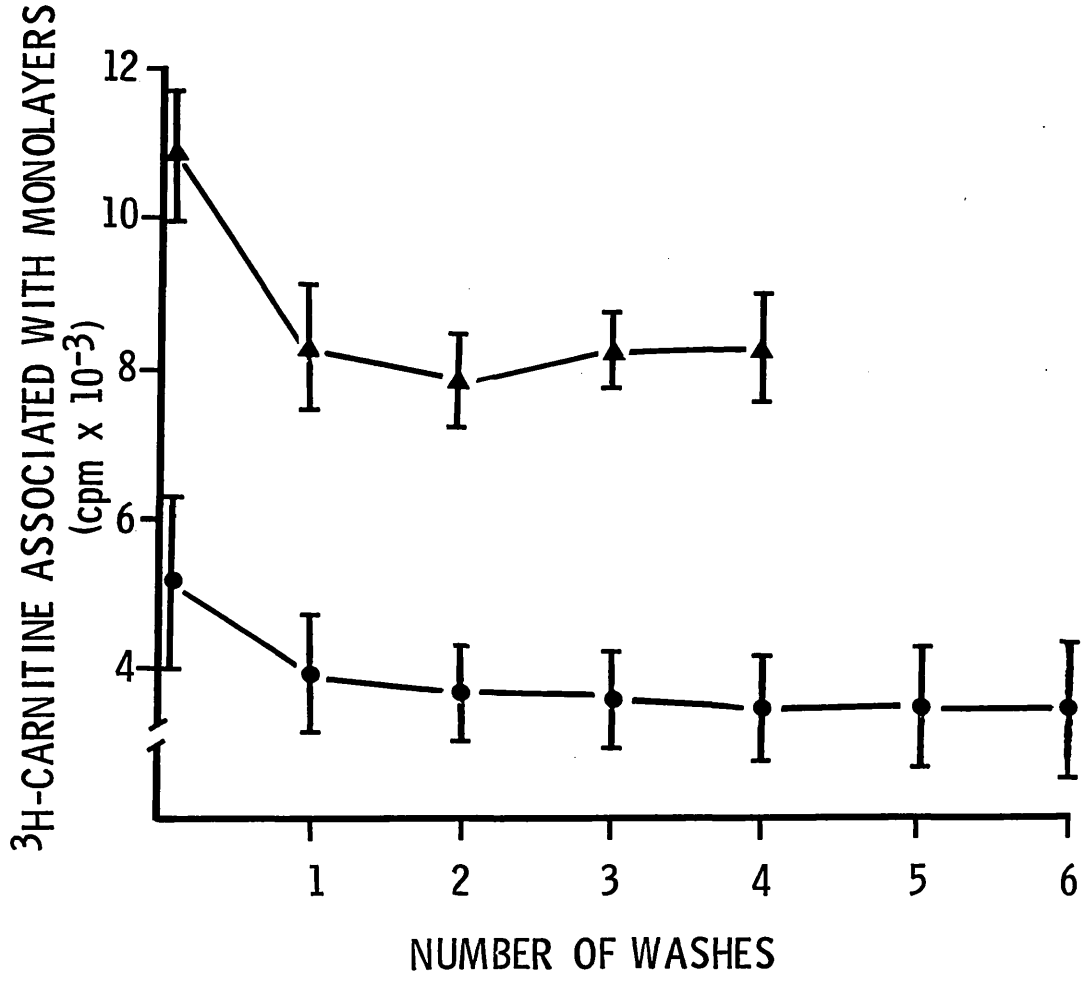


Figure III.3.2.ii

The same experimental procedure was followed as is represented in Figure III.3.2.i, however, in this experiment, incubation was continued for 20 hours.

(ii) Results. The mean counts per minute (cpm ± one standard deviation (SD)) retained in four replicate HFM cultures after successive washes are represented in Figs III.3.2.i and III.3.2.ii.

(iii) Discussion. As can be seen from Figs III.3.2.i and III.3.2.ii, the recovery of ^{3}H-C fell markedly over the first two washes, but succeeding washes did not appreciably lower carnitine recovery from the monolayers. Between 40% and 60% of the monolayer associated ^{3}H-C was removed by the first two washes. In addition, the SD decreased proportionately with the number of washes, however began to increase after 4 washes. It would seem that this removable ^{3}H-C non-specifically associated with the plasma membranes of the cultures, and would have to be removed before any accurate measurement of ^{3}H-C uptake by such cultures could be made. It was therefore decided to wash monolayers three times with culture medium before solubilization for estimation of retained ^{3}H-C. The number of washes used was a compromise between the ability to remove most of the non-specifically associated ^{3}H-C and inflicting minimum damage on the cultured cells by the washing process.

CHAPTER 4

III.4 ^{3}H-CARNITINE UPTAKE WITH TIME.

III.4.1 Introduction

In the previous chapter, it was shown that extra-cellular carnitine tended to associate with cell membranes of human fetal muscle cultures. This could be effectively removed by three washes with culture medium. All cultures used in subsequent experiments underwent the washing proceedure before harvesting, for estimates of tritium-labelled carnitine (^{3}H-C) retained intracellularly. The experiments described in this chapter were designed to see whether the uptake of ^{3}H-C with time could be accurately measured in cultures of human fetal muscle. As these cultures contain a large proportion of fibroblasts, the rate of uptake by muscle derived cultures was compared with that by confluent monolayers of human skin fibroblasts. The cultures were derived and maintained *in vitro* as previously described (II.1). Tritium-labelled carnitine uptake by cultures of a human heart cell line (Girardi) known to take up carnitine by a specific active transport mechanism (Bohmer, Eiklid and Jonsen, 1977) was compared with that by muscle and fibroblast cultures.

III.4.2 ^{3}H-Carnitine uptake by cultures of human fetal skeletal muscle and skin and Girardi heart cells

(i) Experimental design. Monolayer cultures, in 96 well microtitre plates, of human fetal muscle and human fetal skin fibroblasts (derived

from the same fetal source) and Girardi heart cells were established as previously described (II.2). The growth medium was removed and replaced with 100 µl maintainance medium (MM) containing either 10, 1 or 0.1 µCi ^{3}H-C (specific activity 900 mCi/mMole) per millilitre. At intervals of 2,4,6,8 and 24 hours after the commencement of incubation at 37°C (5% CO_2 in air atmosphere), four replicate wells containing ^{3}H-C at the three different concentrations were harvested for intracellular ^{3}H-C, as described in the previous chapter, III.3. Briefly, the supernatant was removed and the monolayers gently washed with three changes of culture medium. The washed monolayers were dissolved in 6% SDS and transferred to scintillation vials. Each well was washed twice with water and these washings pooled with the solubilised cells in the appropriate scintillation vials. Two millilitres (2 ml) of liquid scintillation fluid were added to all vials which were vortex-mixed, left to stand overnight and the β-emission of each sample measured photometrically. The mean counts per minute (cpm) of each four replicates were calculated. The results of a representative experiment showing uptake of ^{3}H-C by each cell culture type (in cpm) ± one standard deviation (SD) at the three different concentrations of added ^{3}H-C at different times of incubation are represented in Table III.4.2. These results are also shown presented as the cpm due to ^{3}H-C retained by the cultures as a percentage of that added at the start of the experiment, in Figs III.4.2i, ii and iii.

(ii) Results and discussion. When ^{3}H-C was added to parallel cultures of either fetal muscle or skin fibroblasts, the muscle cultures retained the label to a greater extent, at all concentrations of ^{3}H-C

	Hours of Incubation	Counts per minute ^{3}H-Carnitine retained					
		HFM*i		HFF*ii		Girardi	
		mean	SD	mean	SD	mean	SD
A	2	2695	506	825	201	5848	639
	4	3292	856	1625	932	10288	1319
	6	5327	780	2132	322	12486	1154
	8	6376	859	2697	882	17202	1723
	24	16029	2218	3460	245	75417	2920
B	2	402	45	220	64	1387	137
	4	723	60	160	19	2385	179
	6	1179	73	322	78	2816	524
	8	1706	239	827	205	4379	1053
	24	3094	403	623	35	12484	425
C	2	75	7	43	14	155	25
	4	107	3	69	24	196	58
	6	230	65	212	95	364	76
	8	367	98	570	56	551	83
	24	417	20	156	17	1279	334

*i HFM = human fetal muscle.

*ii HFF = human fetal skin fibroblasts.

Table III.4.2

Retention of ^{3}H-Carnitine (mean counts per minute, cpm ± one standard deviation, SD) by thrice washed monolayers of human fetal muscle, fetal fibroblasts and Girardi heart cells after 2,4,6,8 or 24 hours incubation. The mean cpm ± 1SD for 1 µCi (Part A), 0.1 µCi (Part B) and 0.01 (Part C) of ^{3}H-Carnitine (Specific activity 900 mCi/mMole) added at the initiation of the experiment were 603,131 ± 2343, 63,231 ± 999 and 6,825 ± 363 respectively.

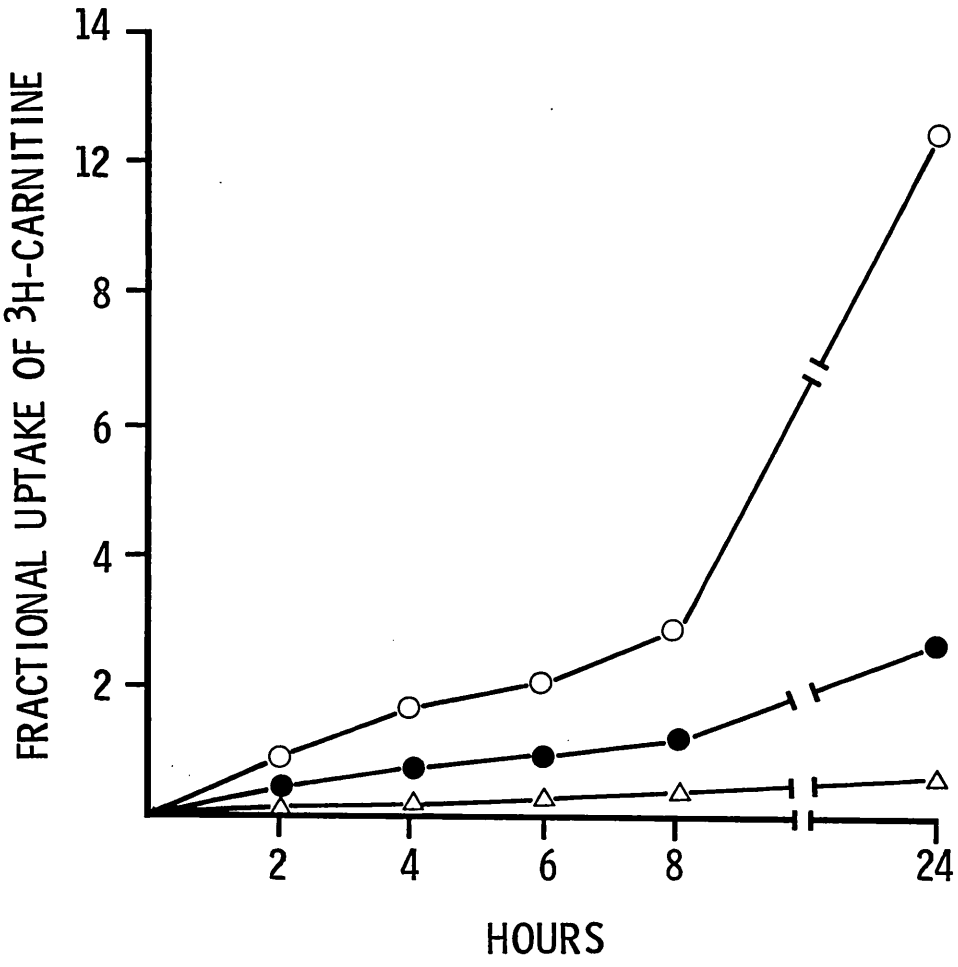


Figure III.4.2.i

The fractional uptake of ^{3}H-Carnitine with time, by monolayers of human fetal muscle (●), human fetal fibroblasts (△) and Girardi heart cells (o), following incubation with 1 µCi of ^{3}H-Carnitine per well. All monolayers were washed thrice with culture medium before harvesting, as described in the text. The mean counts per minute ± one standard deviation of the measurements of ^{3}H-Carnitine retention, on which this plot is based, are given in Table III.4.2.

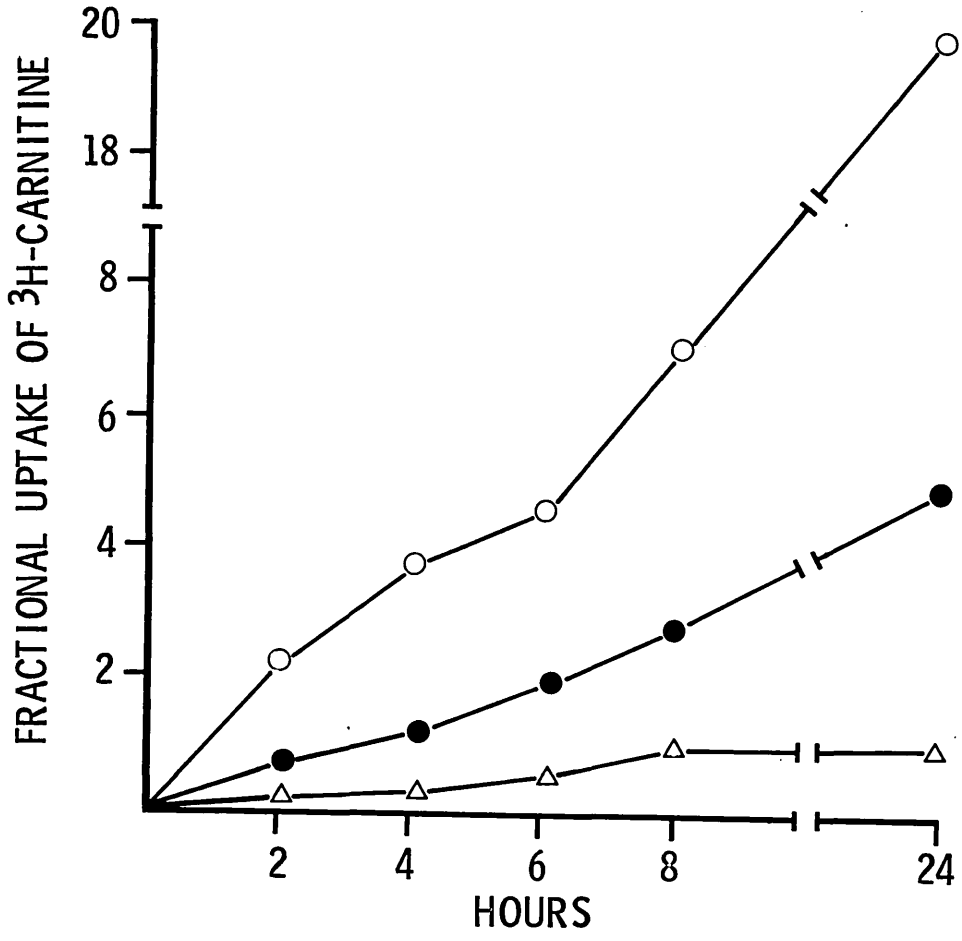


Figure III.4.2.ii

The fractional uptake of ^{3}H-Carnitine with time. Results shown here were obtained and are represented, in the same way as in Figure III.4.2.i. However, 0.1 μCi ^{3}H-Carnitine was added to each well at the initiation of the experiment.

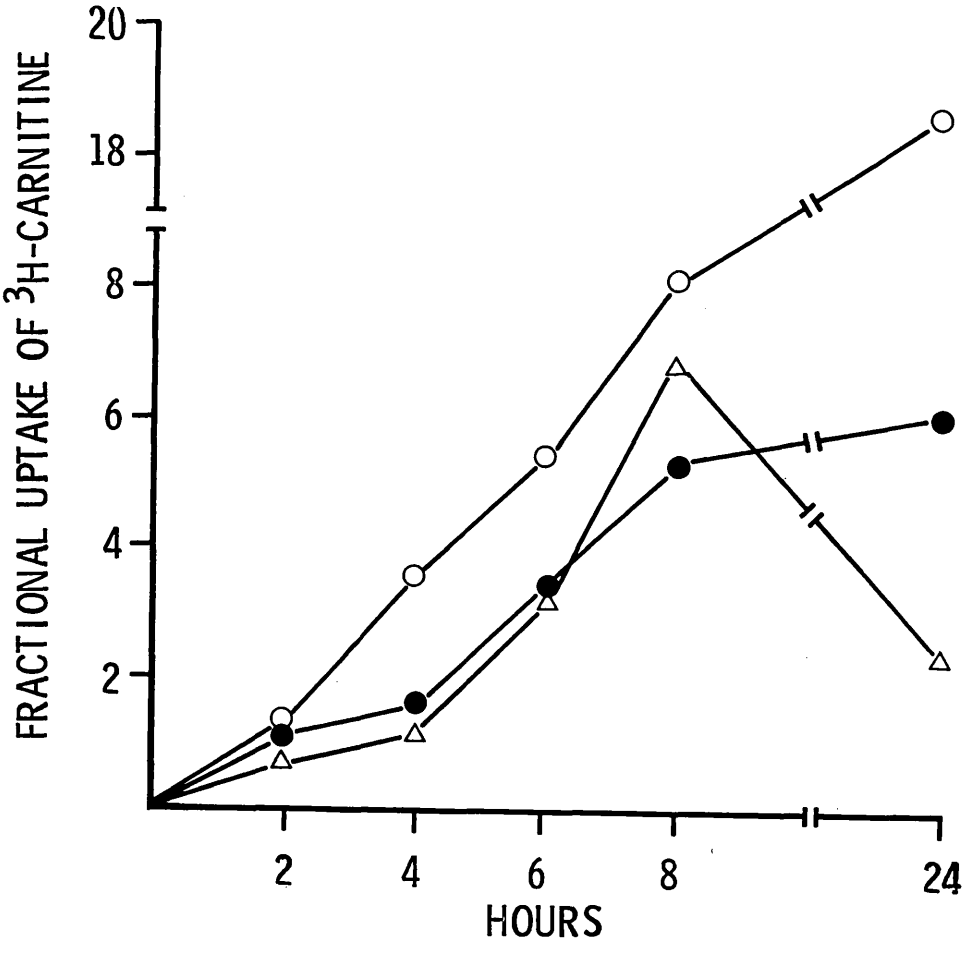


Figure III.4.2.iii

The fractional uptake of ^{3}H-Carnitine with time. The results shown here were obtained and are represented in the same way as those given in Figure III.4.2.i., however, 0.01 µCi ^{3}H-Carnitine was added to each well at the initiation of the experiment.

added (Table III.4.2, Figs III.4.2i, ii, iii). Although myotubes represented only a minority of the cells derived from muscle, their presence was sufficient to create the observed difference in ^{3}H-C retention. The difference between muscle and fibroblast retention was most apparent at an initial concentration of 0.1 µCi ^{3}H-C per well, being 3 fold at 8 hours and 5 fold at 24 hours (Fig.III4.2.ii). The Girardi heart cell cultures, retained an even greater proportion of added ^{3}H-C, however, unlike the human fetal muscle cultures, these cultures consist of an homogeneous population of myocardial-like tumour cells. All three cell types retained a lower proportion of ^{3}H-C added at a concentration of 0.1 µCi/well than at 1.0 µCi/well suggesting that a concentration between 0.1 and 1.0 µCi per well, (0.1 and 1.11 nmoles/well respectively) the active transport system has reached saturation. There was less difference between uptake of ^{3}H-C by the different cell types at 0.01 µCi (0.01 nmoles) per well, Fig.III.4.2iii. Because there was little variation in the proportion of myotubes present in wells cultured from the same fetal source, the fractional retention at diferent times reflected the activity of the membrane associated active transport system for carnitine at different extra- and intracellular molarities of carnitine.

III.4.3 ^{3}H-C uptake by cultures of human fetal muscle and skin fibroblasts

(i) Experimental design. These experiments were designed to measure uptake of ^{3}H-C with time by monolayer cultures of human fetal muscle and syngeneic skin fibroblasts. In each of the eight experiments,

parallel microplate cultures of human fetal muscle and skin fibroblasts were incubated with 100 µl of culture medium containing 0.1 µCi ^{3}H-C per well (specific activity 750 mCi/mMole). This concentration of tritium labelled carnitine was chosen because the previous experiments (III.4.2.) showed that this concentration (0.1 µCi ^{3}H-C/well) was below the saturation concentration for the active transport system. At hourly intervals following the start of incubation at 37 °C (5% CO_2 in air atmosphere), replicates of 4 wells were harvested and retained ^{3}H-C estimated as described in III.4.2.

(ii). Results and discussion. There was considerable variation in the number and size of myotubes between muscle cell cultures derived from different fetal sources. However, there was little variation between cultures in each microtitre plate, derived from the same fetal source. As a result, it was necessary to normalise data obtained from different experiments (Fig III.4.3). The results were normalised to a value of 1 for HFM at 5 hours incubation. In this way, it was possible to pool information from the eight experiments. As can be seen from Fig III.4.3, approximately five times as much ^{3}H-C was recovered from muscle cultures as from fibroblast cultures at each interval of incubation. Fibroblasts took up ^{3}H-C steadily, but more slowly and the larger standard error for fibroblast uptake reflects the smaller amount of labelled compound recovered. The fractional values for the recovery of ^{3}H-C were subjected to regression analysis, the best fit obtained being that a power curve, where $y = ax^{b}$. The values for muscle cultures being $y = 0.7x^{0.3}$ ($R = 0.92$) and for fibroblasts, $y = 0.13x^{0.49}$ ($R = 0.84$). Retention increased progressively over the time period studied, although an incubation period between 10 and 15 hours was

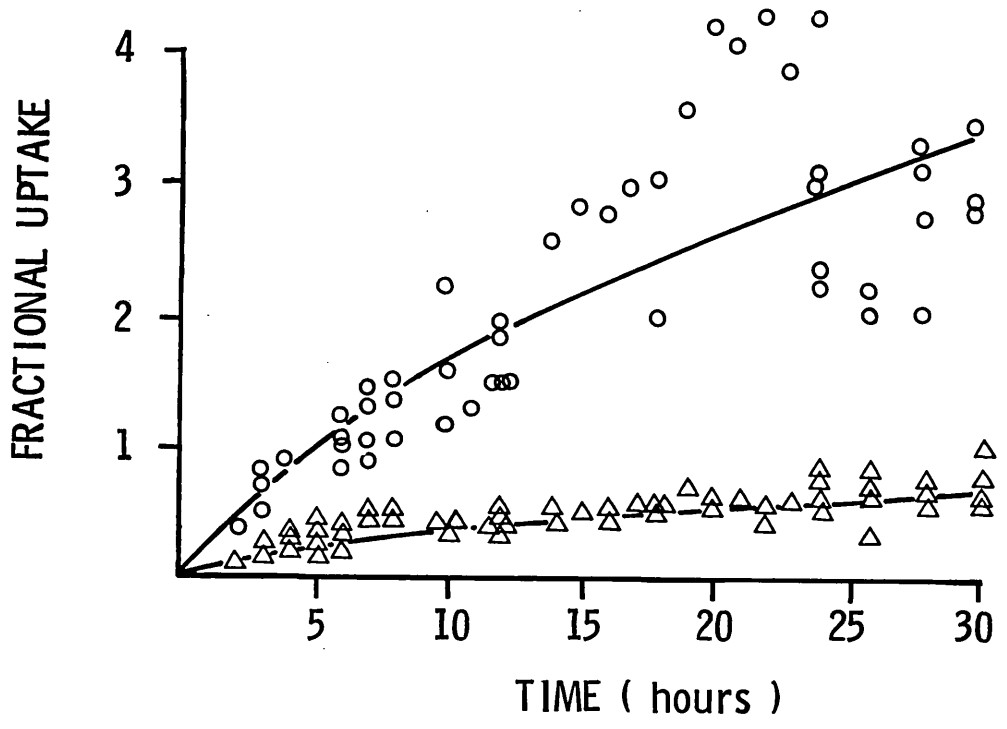


Figure III.4.3

The fractional retention by H-Carnitine labelled muscle cultures (o) and by fetal fibroblast cultures (△) over 30 hours of incubation in a total of 8 separate experiments. The retention of ^{3}H-Carnitine in each experiment has been normalised to a value of 1 for muscle retention at 5 hours in the same experiment. The uptake for ^{3}H-Carnitine followed a power curve $y = ax$ (the best fit for both types of monolayer by linear regression is shown). For muscle cultures this was $y = 0.7x^{0.3}$ ($R = 0.92$) and for fibroblasts, $y = 0.13x^{\cdot 49}$ ($R = 0.84$). Standard errors of the mean were 0.25 and 0.27 respectively. Each value represents the mean of 4 replicate wells after increasing intervals of incubation. The specific activity of ^{3}H-Carnitine used in these experiments was 750 mCi/mMole.

sufficient to keep counting errors below 5%.

III.4.4 Random variation of ^{3}H-C labelling technique

(i) Experimental design

Replicate cultures of HFM, grown in microtitre plates were incubated with 0.1 µCi in 100 µl MM (specific activity of the carnitine used being 900 mCi/mMole) and tritium retained by individual monolayer cultures estimated as previously described (II.3).

(ii) Results and discussion

Table III.4.4 gives the mean value of ^{3}H-C retained by monolayer cultures in five separate experiments. The standard deviation and coefficient of correlation were also calculated and are given in this table as measures of the variability of well to well estimations of ^{3}H-C retained. It can be seen that the standard deviation was consistently less than 15% of the mean values (coefficient of correlation <0.15). This variation between individual estimates was remarkably low when considering the number of technical manipulations involved in establishing, labelling and harvesting HFM monolayers for these experiments. This would seem to imply that ^{3}H-C is taken up in a comparable manner by replicate HFM cultures derived from the same fetus.

Number of wells	mean cpm	SD	coefficient of variation
57	1765	253.2	0.1435
60	2112	195.6	0.0943
16	1996	228.4	0.1295
20	2260	335.2	0.1480
16	2061	167.2	0.0801

Table III.4.4

The well to well variation in labelling of HFM monolayer cultures with ^{3}H-C. The mean and standard deviation (SD) of replicate cultures are represented for 5 separate experiments. The coefficients of variation for individual experiments are also given.

III.4.5 Discussion

From the experiments described in this chapter, it was established that tritium-labelled carnitine was selectively and actively transported into cultures of human fetal muscle to a greater extent than into cultures of skin fibroblasts. Absolute values of ^{3}H-C uptake by cultures derived from different fetuses varied depending on the proportion of myotubes in the cultures. However, the rate of uptake within individual experiments, using material derived from the same fetal source, followed a similar distribution to that of experiments where data was pooled. When results from different experiments were normalised, in the way described (Fig.111.4.3), ^{3}H-C uptake was shown to be similar for each culture, independently of the quantity of myotube plasma membrane present in different experiments. It was therefore possible to establish and label fetal muscle in a reproducible way with ^{3}H-C.

CHAPTER 5

III.5 KINETICS OF CARNITINE UPTAKE BY CULTURES OF HUMAN FETAL MUSCLE AND SKIN FIBROBLASTS

III.5.1. Introduction

The experiments described in this chapter were designed to further investigate the parameters of ^{3}H-C uptake by monolayer cultures of human fetal muscle and skin fibroblasts. The effect of the presence of various molarities of unlabelled carnitine upon the uptake of a constant dose of ^{3}H-C was measured to give a more precise estimate of the concentration of extracellular carnitine required to saturate the membrane associated active transport system. These experiments comprise III.5.2. The first experiments measured uptake of ^{3}H-C by HFM in the presence of increasing molarities of unlabelled carnitine following 2,4,5 and 6 hours incubation, (Tables III.5.2.i and III.5.2.ii and Fig III.5.2.1. The experiments shown in Table III.5.2.iii) and Fig III.5.2.ii), compared the uptake of ^{3}H-C by skin fibroblasts as well as muscle cultures following incubation for 20 hours over a wider range of unlabelled carnitine concentrations.

In III.5.3 of this chapter, the effect of increasing molarities of ^{3}H-C on the rate of carnitine uptake by muscle and fibroblast cultures was measured in order to derive the rate constants, Km, Vmax and KM/Vmax for the carnitine transport system. Conventionally, a comparison between carnitine retained within the cells and that concentration added to the culture medium can be used to derive this information by

double reciprocal regression analysis; a technique known as a Lineweaver-Burke plot.

III.5.2. Rate of uptake of ^{3}H-C in the presence of different molarities of unlabelled carnitine.

(i) Experimental design. The effect of the presence of increasing molarities of unlabelled carnitine on the uptake of a constant concentration of labelled compound was investigated. The ratios of unlabelled to ^{3}H-C labelled carnitine and the total nanomoles (nmoles) added per well in a volume of 100 µl per well are given in Table 5.2.i. At intervals of 2,4,5 and 6 hours after the addition of ^{3}H-labelled carnitine or cold plus labelled carnitine, and incubation at 37°C in 5% CO_2 in air, replicates of 4 wells were harvested as previously described. The cpm for replicates of 4 wells were averaged and the picomoles (pmoles) of ^{3}H-C retained calculated.

(ii) Results. The results are represented in Table III.5.2.i and Fig III.5.2.i. Table III.5.2.i gives the picomoles of ^{3}H-C retained by the cultures at different ratios of cold to ^{3}H-labelled carnitine, and total nanomoles present in the incubation medium for the four point measurements. Fig III.5.2.i expresses these results as a plot of the rate of uptake (1/V where V= average picomoles carnitine retained) against the ratio of unlabelled:labelled carnitine. Straight line regression analysis (Table III.5.2.ii) using the formula $y = ax+b$, (where $y = 1/V$ and x = nmoles added) gave R values >0.96, indicating that the rate of uptake was linear for the extracellular concentrations of carnitine for all four time points.

Nanomoles Carnitine Added		Ratio*	Picomoles ^{3}H-C retained (V) Hours of Incubation			
Unlabelled	Total		2	4	5	6
0	0.0588	0	0.2754	0.4263	0.3822	0.4273
0.025	0.0838	0.425	0.2176	0.2705	0.2538	0.2764
0.050	0.1088	0.850	0.1578	0.2274	0.2293	0.2283
0.100	0.1588	1.701	0.1313	0.1352	0.1245	0.2117

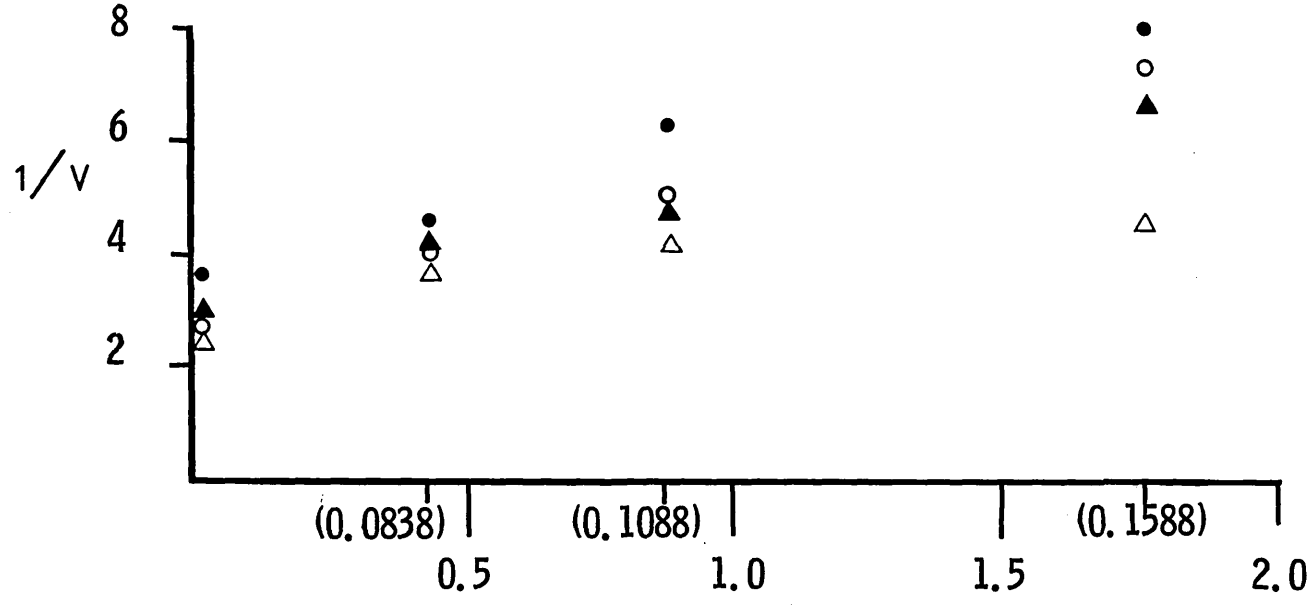
* Ratio = unlabelled:labelled carnitine.

Table III.5.2.i

The picomoles of ^{3}H-Carnitine retained by cultures of human fetal muscle (calculated from the mean counts per minute from 4 replicate wells) following the addition of 0.1 μCi ^{3}H-Carnitine (0.0588 nanomoles) to each well, in the presence of increasing amounts of unlabelled carnitine over different periods of incubation. The specific activity of ^{3}H-Carnitine used was 1.7 Ci/mMole carnitine.

Figure III.5.2.i

The rate of ^{3}H-Carnitine uptake shown on the y-axis (1/V, where V = picomoles of carnitine retained) by monolayer cultures of human fetal muscle following the addition of 0.1 µCi ^{3}H-C and increasing concentrations of unlabelled carnitine to each of 4 replicate wells. Incubation was continued for 2 (●), 4 (○), 5 (▲) or 6 (△) hours. The specific activity of ^{3}H-C used was 1.7 Ci per mMole carnitine. Values for V are given in Table III.5.2.i. On the x-axis is represented the ratio of unlabelled:labelled carnitine added to appropriate wells; figures in parentheses represent total nanomoles carnitine present at each estimation point. Regression analysis of results gave straight lines as best fit (as indicated) and the parameters of these lines are given in Table III.5.2.ii.


8
6
4
2
1/V
(0.0838)
(0.1088)
(0.1588)
0.5
1.0
1.5
2.0
Ratio unlabelled : labelled carnitine

Hours of Incubation	a	b	R	P
2	2.40	3.76	0.97	< 0.01
4	2.92	2.28	0.99	"
5	3.10	2.42	0.97	"
6	1.31	2.78	0.96	"

Table III.5.2.ii

This table gives the values of parameters derived following straight line regression analysis of data in Table III.5.2.i. To obtain this information, results were substituted into the formula $y = ax + b$, where x = total nanomoles of carnitine added and $y = 1/V$ (where V = picomoles ^{3}H-Carnitine retained by cultures) and R is the correlation coefficient. Values of p, the probability for closeness of fit for a straight line, were derived using Student's T-test.

(iii) Experimental design. In this experiment, cultures of human fetal skin fibroblasts, in addition to HFM cultures, were incubated with different ratios of unlabelled to labelled carnitine; however, the ratios used covered a wider range than the previous experiment (see Table III.5.2.i). Cultures were incubated for twenty hours at 37°C in 5% CO_2 in air before harvesting.

(iv) Results. The picomoles of ^{3}H-Carnitine retained by muscle and fibroblast cultures following 20 hours incubation, at different unlabelled:labelled carnitine ratios are represented in Table III.5.2.iii. Figure III.5.2.ii expresses the results as a plot of the rate of ^{3}H-C retention (reciprocal of picomoles retained) against the ratio of unlabelled:labelled carnitine added, in nanomoles per culture.

(v) Discussion. Figure III.5.2.i and Tables III.5.2.i and ii, show the effect of the presence of increasing molarities of unlabelled carnitine on the uptake of a constant dose of ^{3}H-C added per well. When the muscle cultures were incubated for 2,4,5 and 6 hours at nanomolar concentrations of carnitine added varying from 0.059 to 0.158 per well, there was a linear relationship between 1/V (where V = pmols ^{3}H-Carnitine retained by the cultures) and the ratio of 'cold' to ^{3}H-labelled carnitine. This was interpreted to mean that the capacity for carnitine uptake for myotubes was not saturated at this total carnitine concentration. When cultures of human fetal muscle and skin fibroblasts were incubated for 20 hours with total carnitine concentrations ranging from 0.133 and 1.133 nmoles per well, (Table III.5.2.iii and Fig. III.5.2.ii) 1/V reached a plateau at a total carnitine concentration of approximately 0.8 nmoles per well (ratio

Nanomoles Carnitine Added		Ratio*	Picomoles ^{3}H-C retained (V)	
Unlabelled	Total		Fibroblasts	Muscle
0	0.1333	0	2.12	4.68
0.125	0.2583	0.94	1.04	1.86
0.25	0.3833	1.88	0.67	1.50
0.50	0.6333	3.76	0.53	1.15
0.75	0.8833	5.64	0.49	0.94
1.00	1.1333	7.50	0.50	1.01

* Ratio = unlabelled:labelled carnitine

Table III.5.2.iii

The picomoles of ^{3}H-Carnitine retained (V) by human fetal muscle and fibroblast cultures, following incubation of monolayers with 0.1 µCi ^{3}H-Carnitine of specific activity 750 mCi/mMole, (thus containing 0.1333 nanomoles of carnitine) and increasing concentrations of unlabelled carnitine for 20 hours.

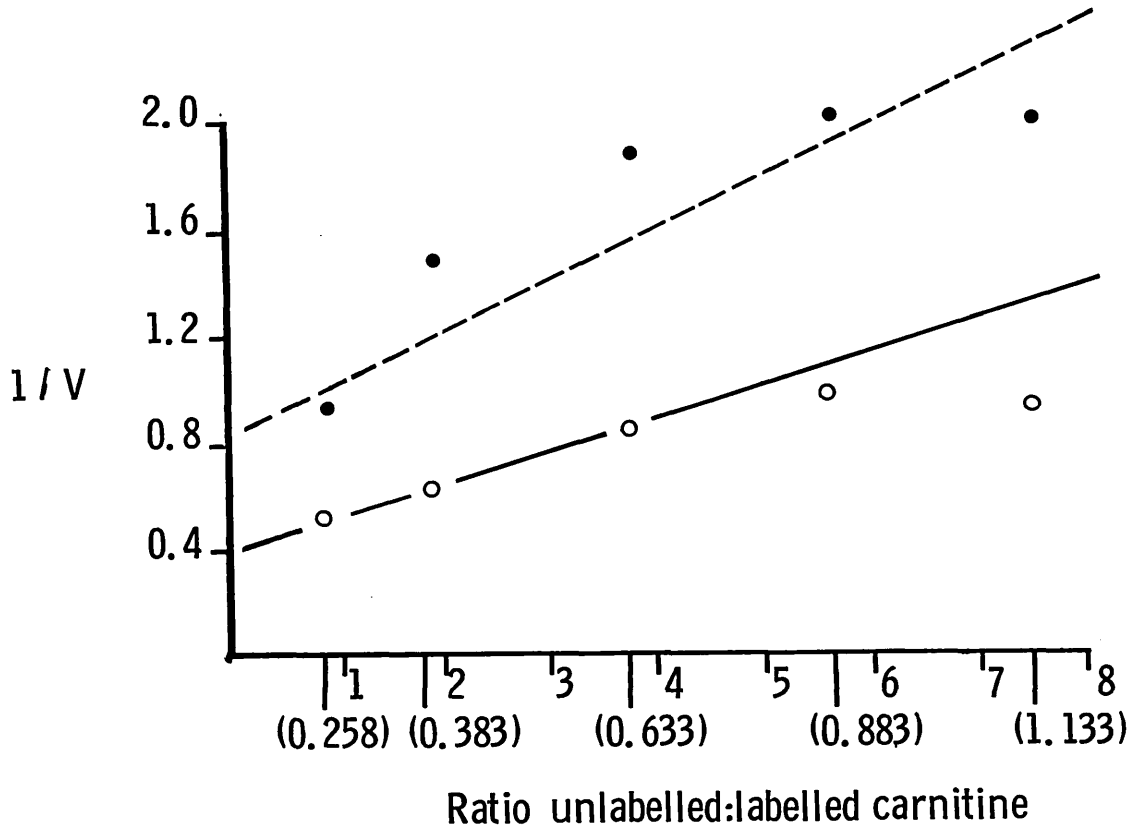


Figure III.5.2.ii

The rate of ^{3}H-Carnitine uptake by monolayers of human fetal muscle (●---●) and fibroblasts (o—o), where 1/V = the reciprocal of picomoles of carnitine retained by cultures. The ratio of unlabelled to labelled carnitine added is represented on the x-axis, with figures in parentheses showing the total nanomoles present for each measurement. Cultures were incubated for 20 hours with 0.1 µCi ^{3}H-Carnitine (specific activity 750 mCi/mMole) and increasing concentrations of unlabelled carnitine. The values for V in this experiment are given in Table III.5.2.iii. Straight line regression analysis, using the formulae $y = ax + b$, where x = ratio of unlabelled to labelled carnitine and y = 1/V gave the solutions $y = 0.01x + 0.39$ with $R = 0.91$ for muscle; and $y = 0.19x + 0.82$, $R = 0.89$ for fibroblasts.

unlabelled:labelled approximately 6 to 1). This suggested that saturation was achieved at 20 hours by this concentration of extracellular carnitine. The 20 hour values for the rate of carnitine uptake by muscle or fibroblast cultures (Table III.5.2.iii) also show that at all concentrations of extracellular carnitine added, the value of V was consistently greater (i.e. 1/V less) for muscle compared with fibroblast cultures.

III.5.3 Lineweaver-Burke plots

(i) Experimental design. Human fetal muscle and skin fibroblast cultures were incubated with 0.0588, 0.1176, 0.1764 or 0.2352 nmoles ^{3}H-C (specific activity 1.7 Ci/mMole carnitine) for 4,6 or 22 hours before harvesting and estimation of ^{3}H-C recovery at each concentration and time point.

(ii) Results. Plots of the reciprocal of the rate of uptake (1/V, where V = picomoles ^{3}H-Carnitine retained) against the reciprocal of the concentration of ^{3}H-C added (1/[S]) are given in Figs III.5.3.i,ii, and iii. Such plots are known as double reciprocal Lineweaver-Burke plots, from which the rate constants Km, Vmax and Km/V can be derived for carnitine uptake by the cultures. Regression analysis values are shown in Table.III.5.3. The Km for muscle remained low, between 0.0577 and 0.0869, while Km for fibroblasts rose from 0.0865 to 0.8547. Vmax for muscle was higher than fibroblasts except for the 22 hour value, when they were similar (5.00 for fibroblasts and 4.17 for muscle) for both types of monolayer. However, Km/V (1/slope) was consistently higher for muscle cultures compared with fibroblast cultures; rising

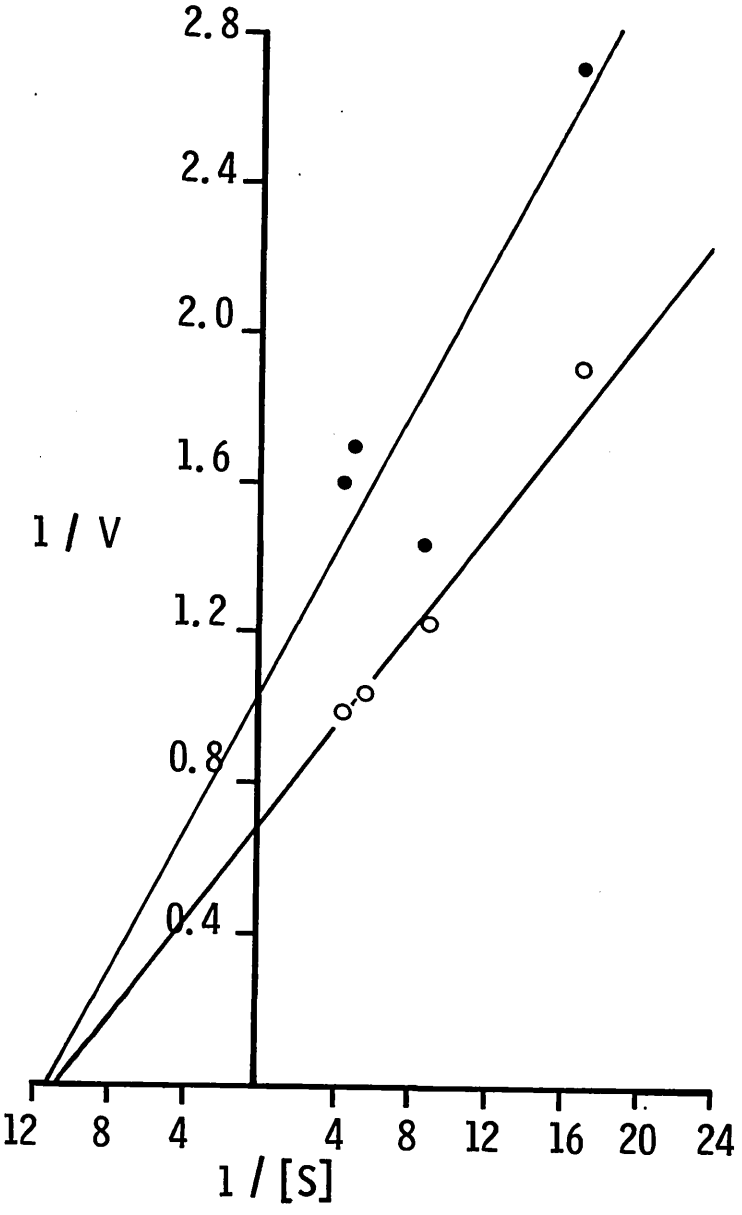


Figure III.5.3.i

Double reciprocal (Lineweaver-Burke) plots for human fetal fibroblasts (o) and muscle (o) monolayers. On the y-axis is represented 1/V (where V = picomoles of ^{3}H-Carnitine retained by monolayers) and on the x-axis, 1/[S], where S is the concentration of ^{3}H-Carnitine added at the initiation of the experiment; being 0.0588. 0.1176, 0.1764 and 0.2352 nanomoles of ^{3}H-Carnitine at a specific activity of 1.7 Ci/mMole carnitine. The parameters for the regression lines represented here are given in Table III.5.3. Incubation was continued for 4 hours in this experiment.

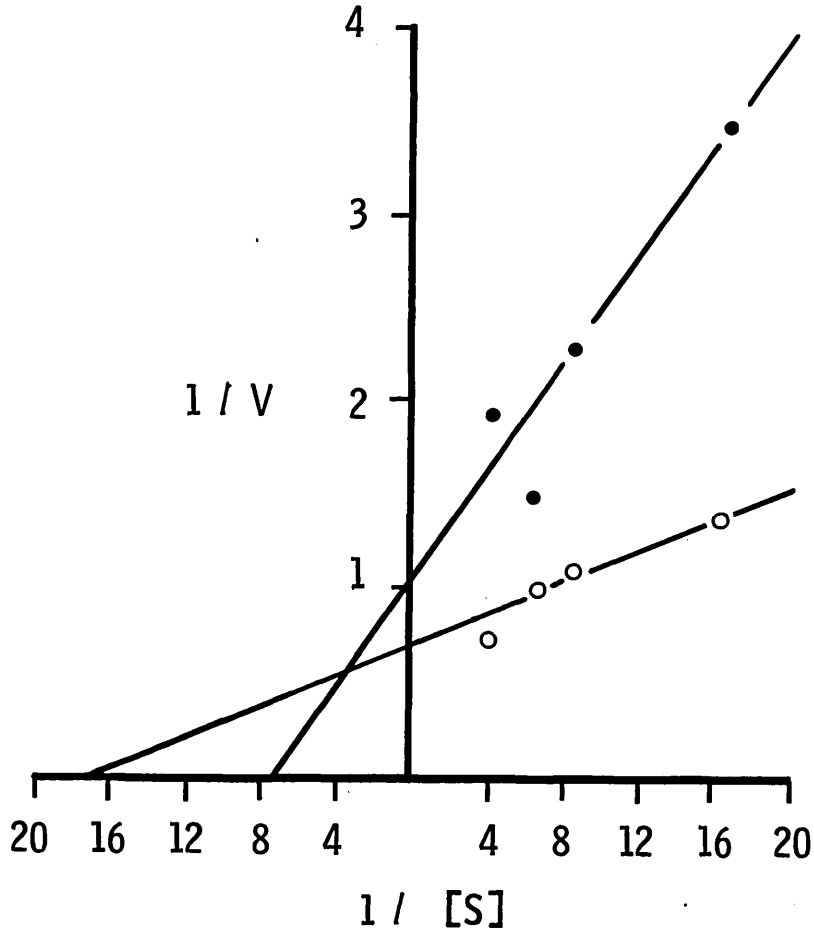


Figure III.5.3.ii

As for Figure III.5.3.i, however, incubation continued for 6 hours.

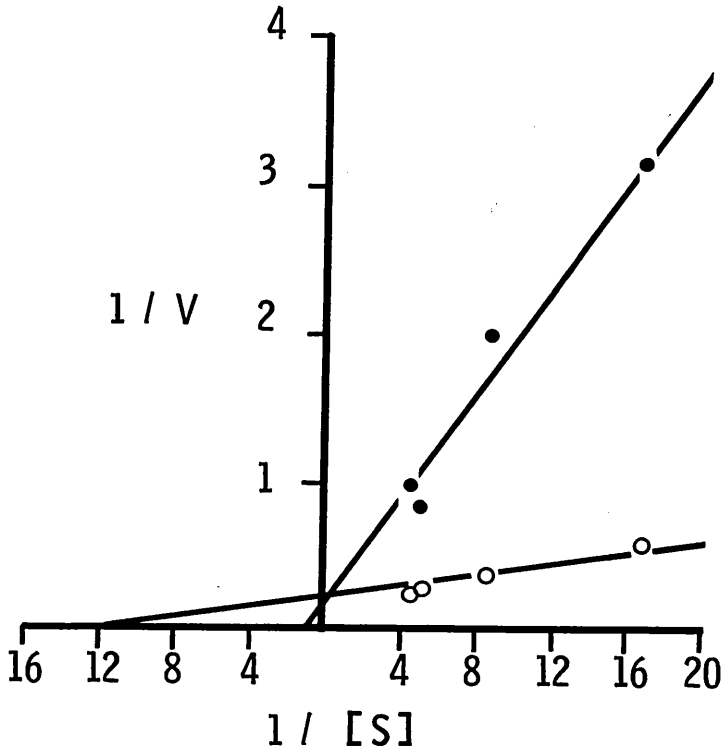


Figure III.5.3.iii

As for Figure III.5.3.i, however, incubation was continued for 22 hours.

Paremeters	Hours of Culture					
	4		6		22	
	**HFF	*HFM	HFF	HFM	HFF	HFM
a	0.09	0.06	0.14	0.04	0.17	0.02
b	1.04	0.69	0.04	0.69	0.20	0.24
SEM	0.48	0.20	0.58	0.10	0.38	0.50
Km	0.0865	0.0869	0.1387	0.0577	0.8547	0.0833
Vmax	0.9615	1.44	0.9901	1.45	5.0	4.17
Km/V(1/a)	11.11	16.67	7.14	25	5.88	50
x-intercept (-1/Km)	-11.56	-11.5	-7.21	-17.25	-1.17	-12
y-intercept (1/Vmax)	1.04	0.69	1.01	0.69	0.20	0.24
R	0.88	0.99	0.95	0.98	0.98	0.99

**HFF = human fetal fibroblasts

* HFM = human fetal muscle

Table III.5.3

Linear regression parameters and values for Km and Vmax for double reciprocal plots of ^{3}H-Carnitine retention by fetal muscle and fibroblast cultures, from the data shown graphically in Figures III.5.3.i, ii and iii. Values for linear regression are given for the formula $y = ax + b$, where $x = 1/[S]$, and S is the concentration of ^{3}H-Carnitine in nanomoles added to wells and $y = 1/V$ where V is the picomoles of ^{3}H-Carnitine retained by cultures. R is the correlation coefficient and SEM is the standard error of the mean for measurements; 4 replicate wells being used for each determination.

from 1.5 fold at 4 hours to nearly 10 fold higher after 22 hours incubation.

(iii) Discussion

These values for Km and Km/V for muscle compared with fibroblast cultures reflect the presence of an active transport system for carnitine in myotube membranes. ^{3}H-C uptake became more efficient as the period of incubation increased (Km/V increased with time) but, as Vmax for fibroblasts also increased at 22 hours, optimal differences in ^{3}H-C uptake between myotubes and fibroblasts at the molarity and specific activity used would be achieved after 10-15 hours of incubation. These experiments further illustrated the efficiency in carnitine uptake by muscle cultures, even though they contained a minority of myotubes amongst a great number of fibroblasts homologous with the cells in the skin fibroblast cultures.

CHAPTER 6

III.6 LOSS OF ^{3}H-CARNITINE FROM PRE-LABELLED HUMAN FETAL MUSCLE MONOLAYERS

III.6.1 Experimental design

In representative experiments described in this chapter, the loss of ^{3}H-Carnitine from pre-labelled cultures of human fetal muscle was investigated. HFM monolayers were incubated with 0.1 µCi ^{3}H-C (specific activity 1.7 Ci/mMole carnitine) for 15 hours. The monolayers were then washed gently with 3 changes of culture medium and incubation continued for up to 28 hours in the presence of culture medium, supplemented with 1% bovine serum albumin, with or without addition of unlabelled equimolar carnitine (0.0588 nanomolar). At intervals following re-incubation, replicates of 4 cultures were harvested and ^{3}H-Carnitine estimated as previously described.

III.6.2 Results

The results of two experiments, showing loss of ^{3}H-C by cultures of HFM in the presence and absence of unlabelled carnitine are shown in Figure III.6.2.

In Experiment I, (solid lines in Figure III.6.2), the value for mean cpm ± 1SD for ^{3}H-C retention were 3,689 ± 243, and for Experiment 2 (dotted lines) 2,483 ± 206 after 15 hours incubation. The difference in mean cpm between these experiments reflected the different fetal

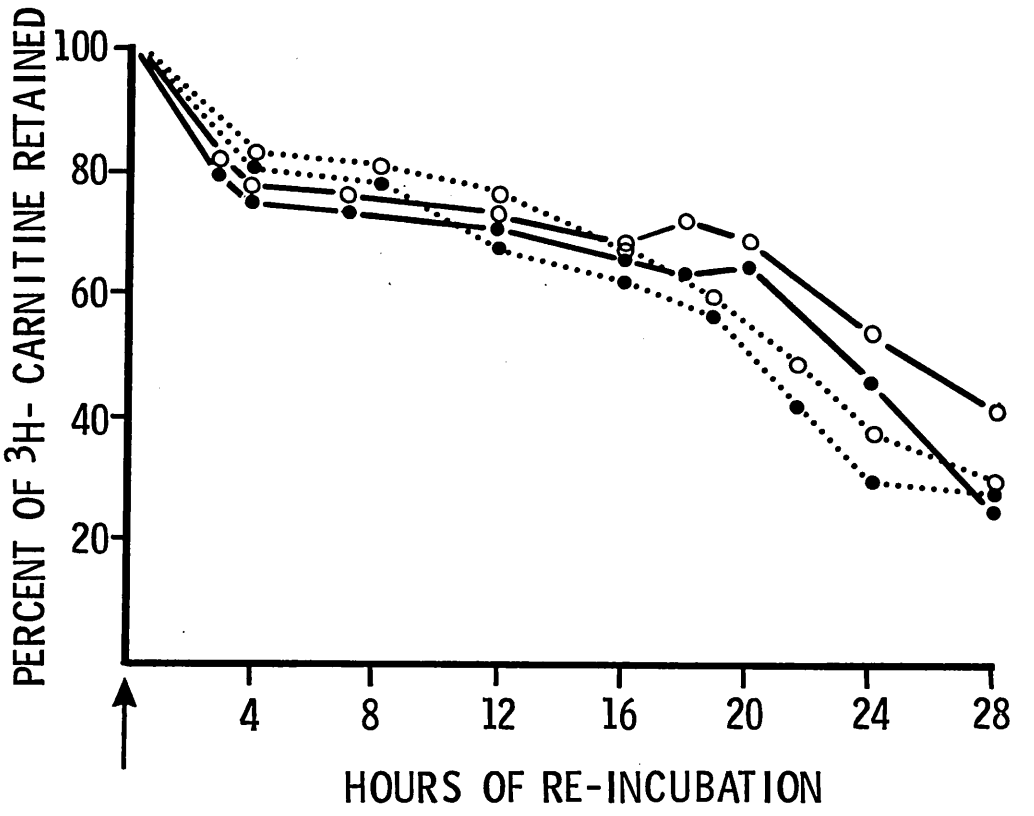


Figure III.6.2

The mean counts per minute (cpm) retained by monolayers after 18 hours incubation is represented as 100% ^{3}H-C retention and Time 0 indicated with arrow. At increasing intervals after replacement of the isotope containing supernatant, mean cpm retained by monolayers of HFM were determined and are expressed as a percentage of the Time 0 value.

sources from which cultures were derived, however, there was considerable similarity between percentage ^{3}H-C retained over the course of the experiments. In addition, it can be seen from Figure III.6.2 that there was very little difference between percentage of ^{3}H-C retained by monolayers of HFM in the presence or absence of extracellular carnitine up to 20 hours after re-incubation. The loss of ^{3}H-C from cultures appeared to be tri-phasic; with a rapid loss in the first 4 hours of incubation, followed by a 'plateau', when further loss was relatively constant and finally a marked decrease in retention approximately 18 hours after re-incubation was begun.

III.6.3 Discussion

From these two representative experiments, it was apparent that the ^{3}H-C content of pre-labelled human fetal muscle monolayers was relatively constant for between 4 and 18 hours after removal of labelled carnitine, regardless of the presence of extracellular carnitine. As these experiments were designed to explore the possibility of using ^{3}H-C as a specific muscle cell label for *in vitro* measurement of muscle cell damage, the spontaneous loss of this compound from cultures should be predictable over the course of such assays. ^{3}H-C loss was less than 20% up to 18 hours after removal of extracellular label, and the loss curve plateaued between 4 and 20 hours after re-incubation. In addition, as it was planned to use fetal calf and human serum in immunological assays, both of which contain approximately 33-54 nano-moles of carnitine per ml, (Marquis and Fritz, 1965), the finding that a similar concentration of unlabelled carnitine in the culture supernatant did not affect ^{3}H-C loss from prelabelled muscle cultures

was also pertinent to such projected studies.

CHAPTER 7

III.7 RADIOAUTOGRAPHY

III.7.1 Introduction

The experiments reported in the previous chapters of the section have examined the specificity, and kinetics of ^{3}H-Carnitine uptake for cultures of human fetal muscle. In order to visualise the distribution of ^{3}H-C in the mixed cell cultures derived from disaggregated fetal muscle, radioautography of ^{3}H-C labelled muscle cultures was performed as described in II.4.4.

III.7.2 Results and Discussion

Carnitine is a small, rapidly-diffusing molecule and it was thus necessary to freeze-dry the labelled monolayers after washing. As can be seen in Figure III.7.2, the morphology of the monolayers was well-preserved, although crystals of culture medium, used as the washing material, remained on the surface and led to some distortion of the photographic emulsion with faint, non-specific silver precipitation. However, the inter-myotubular spaces, which contained fibroblasts, can be seen to be almost free of silver deposition. Tritium-induced silver deposition was largely confined to the multi-nucleate myotubes. Quantitation was not attempted, firstly because the multicellular nature of the cultures made it difficult to relate myotube uptake to that by the smaller, although more numerous fibroblasts. In addition, designation of representative areas of the monolayer, for comparative

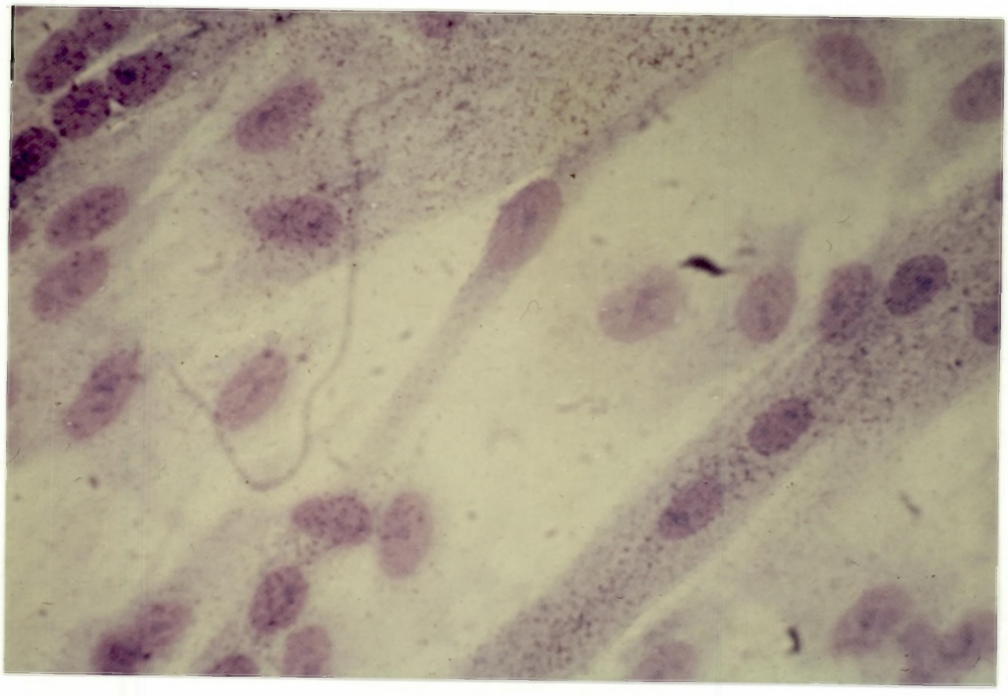


Figure III.7.2

Photomicrograph, taken from a colour transparency of a freeze dried fetal muscle monolayer which had been incubated with ^{3}H-Carnitine. Silver grain deposition is seen closely associated with the large multi-nuclear myotubes. Fibroblasts, seen in the inter-myotubular spaces, are almost free of precipitated silver. (Magnification x200).

counting might have introduced an element of bias, as some areas had relatively greater of fewer myotubes to fibroblasts.

In conclusion, studies of radioautography in ^{3}H-Carnitine labelled fetal muscle cultures provided technically-independent confirmation of the preferential uptake of ^{3}H-Carnitine by myotubes in these cultures.

SECTION IV

CLINICAL STUDIES

IV CLINICAL STUDIES

CHAPTER 1

IV.1 INTRODUCTION TO CLINICAL STUDIES

IV.1.1 Introduction

The clinical presentation of inflammatory muscle disease (IMD), which includes polymyositis and dermatomyositis, is pleomorphic in that patients may show strikingly different patterns of disease at diagnosis. This may in part be due to the possiblity that IMD represents an heterogeneity of disease, or that varying degrees of initial severity allow patients to present at different stages of the disease. Some patients, on detailed investigation, have been shown to possess an underlying immunological or other disorder; for example, complement deficiency, hypogammaglobulinaemia, or chronic viral myositis.

As previously discussed in Section I, it is generally agreed that effector lymphocytes directed against muscle antigens are of primary importance in the pathogenesis of IMD. If IMD is the consequence of activated lymphocytes directed against and capable of damaging muscle, quantitation of this immune response could be used as a measure of disease activity, which would be of potential value in the clinical management of cases. Furthermore, it is possible that the test might distinguish different types of IMD. Although many patients may have IMD as a consequence of immune responses against muscle, they cannot at

present be categorized.

IV.1.2 Previous studies of in vitro myotoxicity

The studies in the following chapters of this section were initially undertaken with several questions in mind:

1. Is specific lymphocyte-mediated myotoxicity pathognomonic for IMD?

2. If it is, can myotoxicity be quantitated accurately and used as a measure of disease activity in IMD patients?

Previous studies have shown that lymphocytes from patients with IMD were more damaging to cultures of both animal and human muscle than control lymphocytes (Dawkins and Mastaglia, 1973). However, the tests of myotoxicity used suffered from one common problem - lack of specificity for muscle cell damage.

When muscle is disaggregated and grown in culture, two cell types develop. As well as the outgrowth of myoblasts which subsequently fuse to become multinucleate myotubes or pre-muscle cells, a large number of rapidly dividing fibroblasts are also present. Conventional measures of target cell recognition and damage by effector lymphocytes have been applied to myotoxicity assays, in both EAM and human IMD, with the following problems:-

(a) Dye exclusion: Direct observation of muscle cultures after incubation with lymphocytes and subsequent staining with intra-vital

dyes. It is difficult to tell which cells in the monolayer have been damaged, and the results are thus difficult to quantitate, (Kakulas, 1966).

(b) Cytotoxicity studies based on Chromium-51 release: Fibroblasts present in muscle cultures also take up Chromium-51 (^{51}Cr), thus the proportion taken up by myotubes is relatively small. Consequently, when myotube killing is greatest, there is only a small difference between non-specific and specific release with respect to myotube killing, but a much larger difference between specific release and maximum release.

(c) Immune adherence

The adherence of peripheral blood lymphocytes from guinea pigs with EAM, to monolayer cultures of chick skeletal muscle has been used as an indicator of specific muscle antigen recognition. The numbers of myotube-adherent cells present in these lymphocyte populations are compared with those present in populations from control animals (Partridge and Smith, 1976). The major disadvantage of this approach is that it gives no information about the ability of adherent lymphocytes to damage cultured muscle cells.

It is clear from examining the assays available for measuring immune responses against muscle, that an in vitro test, which was specific with respect to muscle cell damage, was required before accurate quantitation of myotoxicity could be made.

IV.1.3 The potential of tritium-labelled carnitine as a specific muscle cell label

The most appropriate immunological test for measuring immune responses against cell membrane antigens is that of cytotoxicity. In the case of (IMD), conventional cytotoxicity assays, based on the release of ^{51}Cr as an indicator of muscle cell damage, are neither specific nor sensitive, because of the mixed cell nature of muscle cell cultures.

The approach taken in the following experiments was to employ a compound selectively taken up by myotubes, which would be rapidly released following myotube damage, as the basis for a specific and quantitative myotoxicity assay. Carnitine seemed a promising candidate, being also readily available commercially in tritium-labelled form.

From studies of isolated animal heart, whole muscle and also a cultured human heart cell line, Girardi heart cells, carnitine has been shown to be selectively accumulated by both cardiac and skeletal muscle via a membrane associated active tranport mechanism (Rebouche, 1977; Bohmer, Eiklid and Jonsen, 1977).

However, the physiology of carnitine metabolism by cultures of human skeletal muscle had not been previously reported. It was thus necessary to establish parameters of carnitine uptake and loss by such cultures in order to determine whether carnitine would be a suitable label for muscle cells on which to base a study of lymphocyte-mediated myotoxicity. The results of experiments of the physiology of carnitine

metabolism were thus undertaken, and are described and discussed in Section III. From these studies, carnitine was found to be a suitable specific muscle cell label. Consequently, ^{3}H-Carnitine labelled human fetal muscle cultures form the basis for the following section on clinical studies of *in vitro* myotoxicity by lymphocytes from patients with IMD.

IV.1.4 Problems of studying inflammatory muscle disease in human subjects

There are many difficulties inherent in undertaking a study of patients with inflammatory muscle disease (IMD). The incidence in the general population is approximately 5 per million (Section I), and, although considerable co-operation was given by other large London teaching hospitals, referral of patients for follow-up study proved difficult. Obviously, it was also difficult to control the study with respect to when the patient initially presented with symptoms. Most of the patients had been investigated and treated with immunosuppressive drugs before an opportunity presented itself to include them in the study. However, every effort was made to have suitable muscle cultures available in order to allow experiments to be undertaken at short notice.

Because of the relative rarity of IMD, and in order not to 'miss' any patients as yet not definitely diagnosed, all patients with suspected IMD were studied. When a clinical diagnosis of IMD was confirmed or rejected, the experimental results obtained from these patients were included in the study where appropriate.

IV.1.5 Selection of control subjects

Three groups of control subjects were studied in conjunction with the IMD patients:-

(a) Non-inflammatory muscle disease: This group consisted of patients with congenital muscular dystrophies. They were included to represent a group of patients with muscle disease, but without known immunological involvement in their aetiology or pathogenesis. None of the patients in this group was receiving corticosteroids or other drug therapy.

(b) Connective tissue disease: This group mainly consisted of patients with systemic lupus erythematosus, although some with scleroderma were also studied. These groups of patients were chosen because they represented a disease category known to have immunological abnormalities in association with their disease. These patients were also suitable as a control group with respect to corticosteroid and other immunosuppressive therapy, which most were receiving when studied.

(c) Normal controls: Medical and laboratory staff were, as so often, the most convenient and willing group available for study.

IV.1.6 Clinical details of patients studied

The clinicl details of patients studied in this thesis have been summarised in tabular form. Patients with inflammatory muscle disease

Initials of patient	Age/Sex	Diagnosis	Length of history (years)	Chapters
SA	5M	PM	<1	3 7.3.1 7.3.2 7.3.4
PB	42F	DM	2	7.3.1
VB	56M	DM	3	7.3.1 7.3.2 7.3.4
MC	34F	PM	4	3
BE	44F	PM	4	3
BH	35F	DM	2	3
CH	7F	PM	<1	2
GH	12M	DM	2	4
MH	52M	PM	2	7.3.2 7.3.4
RH	8M	DM	3	3
SH	12M	PM	<1	3 7.3.1 7.3.2

Table IV.1.6.i INFLAMMATORY MUSCLE DISEASE PATIENTS

Initials of patient	Age/Sex	Diagnosis	Length of history (years)	Chapters
EK	37F	DM	3	3 7.3.1
HM	25F	PM/SLE	<1	7.3.1 7.3.2
JM	62M	PM	<1	7.3.4
MM	33F	PM	<1	3
IP	14M	DM	3	2 3
KP	9F	PM	<1	2
ER	7F	PM	<1	3
CS	6F	DM	2	2 4 7.3.1 7.3.2
PS	14M	PM	<1	7.3.1 7.3.3 7.3.4
AW	5F	PM	<1	2 3 4

Abreviations PM: polymyositis
DM: dermatomyositis
SLE: systemic lupus erythematosus

Table IV.1.6.i ctd.

Initials of patient	Age/Sex	Diagnosis	Length of History (yrs)	Activity of Disease	Other Clinical features	Evidence of muscle involvement	CPK
BB	32F	SLE	2	++	Arthralgia	nil	N
MB	29F	Scleroderma	2	diffuse progressive	-	clinically involved	N
	"	"	"	"	"	"	↑
PB	25F	SLE	2	++	Severe skin involvement	nil	N
FC	42F	SLE	4	++	Cerebral involvement	nil	N
MC a)	41F	SLE/PM	1	++	Arthralgia, lung involvement	EMG-normal Myositis in muscle biopsy	N
b)	41F	"	"	++	"	"	N
DH	28F	SLE	2	+	Cerebral involvement	nil	N
HH	35F	Scleroderma	2	CREST non-diffuse	-	nil	N
DJ	30F	SLE/MCTD	1	+	Arthralgia, myalgia	nil	N
AM a)	35F	SLE/PM	10	+++	Lung, cerebral involvement, arthralgia	EMG-myopathic myositis in muscle biopsy	↑↑
b)	"	"	"	++			↑

Table IV.1.6.ii CONNECTIVE TISSUE DISEASE PATIENTS

Initials of patient	Age/Sex	Diagnosis	Length of History (yrs)	Activity of Disease	Other Clinical features	Evidence of muscle involvement	CPK
CM a)	34F	Scleroderma	2	mild,	-	nil	N
b)	"	"	"	CRST	-	"	N
MN	50F	SLE	3	+	Arthralgia	nil	N
NP	40F	Scleroderma	4	CREST, non-diffuse	-	nil	N
PP	35F	SLE	4	++	Arthralgia	nil	N
CT	32F	Sjogren's	1	++	nil	nil	N
JW a)	35F	Scleroderma	3	mild calcinosis	-	nil	N
b)	"	"	"	"	"	"	N

Abreviations* SLE: systemic lupus erythematosus
PM: polymyositis
C: calcinosis
R: Reynaud's sign
E: esosphageal involvement
S: sclerodactyly
T: telangiectasia
CPK: Serum creatine phosphokinase level
N = within normal limits
↑ = elevated to less than twice upper normal limit
↑↑ = elevated to more than twice upper normal limit

Table IV.1.6.ii cont.

Initials of Patient	Age/Sex	Diagnosis
HB	14M	Becker dystrophy
SB	22M	Duchenne muscular dystrophy
JH	9F	Limb girdle dystrophy
MN	12M	Duchenne muscular dystrophy
SP	10M	Duchenne muscular dystrophy
MS	18M	Limb girdle dystrophy

Table IV.1.6.iii CONGENITAL MUSCULAR DYSTROPHY PATIENTS

have been classified as having either polymyositis (PM) or dermatomyositis (DM) according to the criteria of Bohan and Peter (1975), by the clinicians concerned with the care of these patients; the diagnosis of these and the control patient groups being made without the knowledge of the author, whenever possible.

The drug treatment, which patients were receiving when blood was taken for experiments, and the clinical assessment of disease activity, are given for individual patients in the appropriate chapters.

The tables are presented in the following way: Patients are represented by their initials in alphabetical order. Those belonging to the inflammatory muscle disease group are included in Table IV.1.6.i together with the numbers of the chapter(s) in which they appear. The connective tissue disease patient group which were studied for Chapter 5 are given in Table IV.1.6.ii and the patients with congenital dystrophies (Chapter 2) in Table IV.1.6.iii.

In Chapters 5, 6 and 7 of this Section, results of cytotoxicity tests are given in tabular form and also represented graphically. Although it is unusual for results to be represented in two different ways in a thesis, it was thought necessary for reasons of clarity, and to show the distribution of these results in groups of patients and controls.

IV

CHAPTER 2

IV.2 LYMPHOCYTE-MEDIATED MYOTOXICITY - PRELIMINARY STUDIES

IV.2.1 Introduction

This study was undertaken to determine whether any significant difference existed between the myotoxic capacity of lymphocytes from patients known to have active inflammatory muscle disease (IMD) compared with lymphocytes from a group of patients with non-inflammatory muscle disease (NIMD). Myotoxicity tests based on the retention of ^{3}H-Carnitine by cultures of human fetal muscle were used.

The five patients with IMD included in this pilot study were chosen because their disease was considered to be active on clinical and laboratory grounds at the time when blood was taken for estimates of lymphocyte-mediated myotoxicity. Summaries of the clinical details of these patients are included, in the tables given in IV.1.6.

The 6 patients included in the non-inflammatory muscle disease group consisted of 3 patients with Duchenne Muscular dystrophy, 1 with Becker dystrophy and 2 with limb girdle dystrophy, all diagnosed on clinical grounds in the Department of Paediatrics and Neonatal Medicine at Hammersmith Hospital. The patients in both groups were studied within the same six months period.

IV.2.2 Experimental Design

For each estimate of lymphocyte-mediated myotoxicity, ^{3}H-Carnitine labelled human fetal muscle cultures were incubated with lymphocytes from patients in either of the two patients groups. It was also necessary to include a parallel assay of myotoxicity by lymphocytes from a control subject against cultures derived from the same fetus. These lymphocytes were obtained from either of the same two healthy laboratory staff for all assays. It was necessary to include control lymphocyte populations in conjunction with the test lymphocytes, because an 18-hour myotoxicity assay was used in these preliminary experiments. Eighteen hours is a sufficient period of time for cytotoxic precursor cells present in peripheral blood lymphocyte populations to become activated by major histocompatibility complex (MHC) antigens perhaps present on the membranes of allogeneic muscle cell layers. The contribution of natural cytotoxicity due to Natural Killer cell activity to the results is discussed later in this section.

As it was necessary to take into account any activation and cytotoxicity resulting from such an interaction over the period of the assay, by including lymphocytes from normal individuals as a control population, the formula used to calculate % myotoxicity was the following:

$$\% \text{ myotoxicity} = \left(\frac{(C-T)}{M}\right) \times 100$$

where C = Average (n=4) cpm ^{3}H-Carnitine retained following incubation

of HFM with lymphocytes from control subject.

where T = Average (n=4) cpm ^{3}H-Carnitine retained following incubation of the HFM with lymphocytes from the patient.

where M = Average (n=4) cpm ^{3}H-Carnitine retained following incubation of HFM with culture medium alone.

The aim of this preliminary study was to determine whether there was any difference in myotoxic potential between lymphocytes from patients with IMD and those from NIMD patients, so myotoxicity was calculated from values given at a single lymphocyte concentration.

The concentration of 5×10^{5} lymphocytes per well was chosen as appropriate because estimates of the numbers of myotubes per well made by appropriate staining and counting gave values of between 1,000 and 3,000 myotubes per well (personal observation). This would give an approximate effector to target cell ratio of between 500 and 200 to 1. A high effector to target cell ratio was chosen because of the very large surface area of myotubes compared with that of target cells such as K562 or other tumour targets studied in conventional cytotoxicity experiments.

In each assay of myotoxicity, replicate wells of ^{3}H-C-labelled human fetal muscle cultures were incubated with 5×10^{5} lymphocytes from patient or control subjects, and ^{3}H-C retained by the monolayers estimated after 18 hours' incubation at 37°C in 5% CO_2 in air. The details of the method used for this assay are included in Section II.

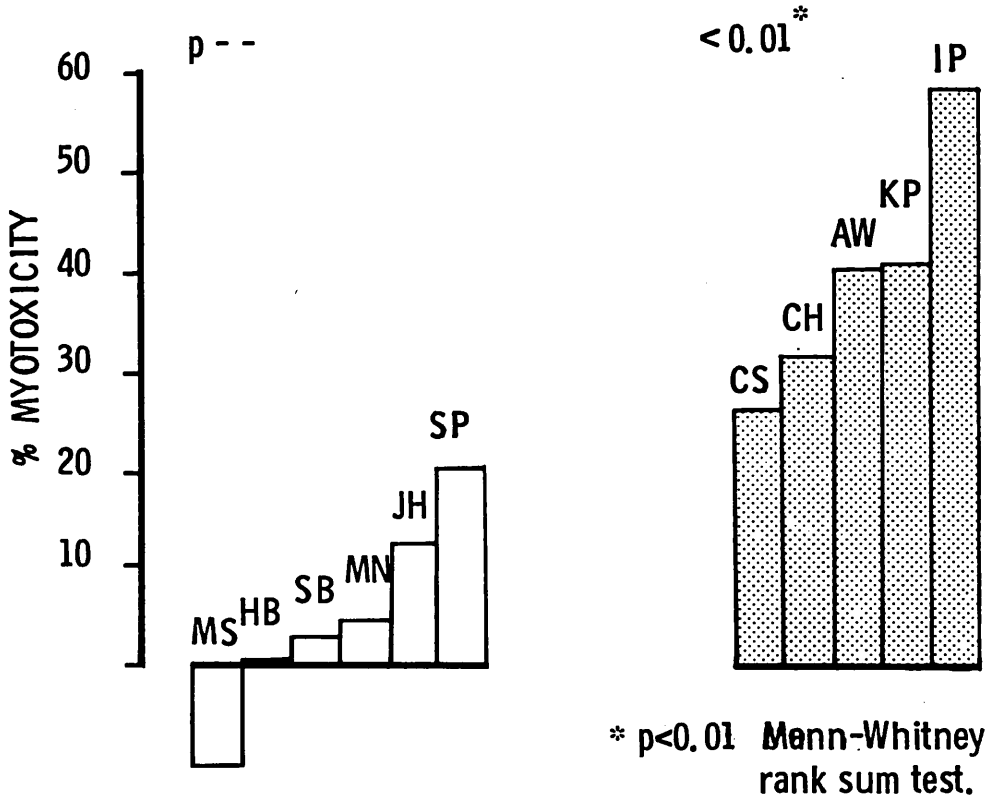


Fig IV.2.3

Percentage myotoxicity shown by lymphocytes at a concentration of 5 x 10^5/well, from 6 patients with non-inflammatory muscle disease (open bars) and 5 with active inflammatory muscle disease (dotted bars) in an 18 hour assay. The initials of the patients studied are depicted above appropriate bars. Drugs being administered at the time of the assays, together with disease activity in the patients, are shown in Table IV.2.3.

Initials of Patient	Disease Activity	*CPK	Treatment Prednisolone (mg/day)
CH	++	↑↑	12
IP	++	↑	nil
KP	+++	↑	10
CS	+	N	15
AW	++	↑	12

*CPK: Serum level of the creatine phophokinase enzyme.

Table IV.2.3

The disease activity of the 5 IMD patients studied in this chapter is represented, relative to that at other times in the course of their illness according to an arbitrary clinical scale; being depicted as follows -'mild' (+), 'moderate' (++) or 'severe' (+++). The CPK values are given as being within normal limits (N) appropriate to the age and sex of the patient, elevated to less than twice the upper normal limit (↑) or more than twice the upper limit of normal values (↑↑).

IV.2.3 Results

The results of this preliminary study are represented in Fig.IV.2.3. Percentage myotoxicity was calculated by the formula given. Values of myotoxicity for the two normal individuals whose lymphocytes served as controls in each assay were therefore zero. Drug treatment, disease activity and CPK values for each IMD patient are given in Table IV.2.3.

Graphically, it can be seen from Fig.IV.2.3 that lymphocytes from all patients with active IMD showed higher absolute values of myotoxicity than those from NIMD patients. The represented data were subjected to Mann-Whitney rank sum analysis, comparing active IMD with NIMD groups, and the difference was significant at $p < 0.01$ level. It is also of note that percentage myotoxicity by lymphocytes obtained from Patient A.W., in whom disease activity at the time of study was regarded as 'mild' compared with other times in the course of her illness, was the lowest value in the group, (Table IV.2.3).

IV.2.4 Discussion

Previous investigations of *in vitro* cytotoxicity by lymphocytes from patients with IMD have reported significantly greater levels of cytotoxicity both against cultures of allogeneic and xenogeneic muscle than that shown by lymphocytes from appropriate control groups. However, quantitative measurements of specific muscle cell (myotube) killing have been hampered by the presence of fibroblasts in such cultures. In section III, tritium-labelled Carnitine (^{3}H-C) was shown to be a selective label for the myotubes present in human fetal muscle

cultures (HFM).

The preliminary studies reported in this chapter were undertaken to determine whether ^{3}H-C-labelled HFM cultures could form the basis of an in vitro test which measured lymphocyte-mediated myotoxicity. Although the numbers of patients studied were small, the results revealed differences in myotoxic ability between lymphocytes form IMD and those from NIMD, which encouraged further investigation. However, other than being an interesting observation, further interpretation of the results from this preliminary study are limited by both technical and statistical restrictions.

(a) Technical: An 18-hour mytotoxicity assay was used in these preliminary studies. High non-specific release of ^{3}H-C from pre-labelled HFM cultures occurred after 18 hours' incubation, increasing background levels, thus reducing the sensitivity of the assay (See III.7).

(b) Statistical: It can be seen from the results shown in Fig.IV.2.3 that although a difference between the two groups of patients was significant at the 1% level when the data were subjected to Mann-Whitney rank sum analysis, the absolute value of the highest value of myotoxicity shown by lymphocytes from a patient with NIMD was only 9% less than the lowest value recorded for lymphocytes from a patient with active IMD. Given the technical restrictions on measurements obtained from the 18 hour myotoxicity test, interpretation of results from experiments on individual patients would prove difficult. A larger number of patients from each group would have to be studied, and a

statistically determined value for percentage myotoxicity of significance thus delineated the calculations based on population statistics for the interpretation of myotoxicity results are not practical, given the rarity of IMD. This manipulation would still not allow comparison of values of myotoxicity by lymphocytes from individual patients on different occasions.

(c) Problems caused by control lymphocytes: An allowance for ^{3}H-C retention by HFM monolayers, after incubation with control lymphocytes from normal individuals, was made when calculating myotoxicity caused by lymphocytes from patients. The reasons for making this allowance are given in the Introduction to this chapter. The amount by which calculated myotoxicity will be affected by values for control lymphocytes will vary between tests and prevent comparison of myotoxicity by lymphocytes from the same patient on different occasions. It is also not possible to calculate myotoxicity on a per lymphocyte basis using this 18 hour incubation period for determining myotoxicity, for the same reason.

IV

CHAPTER 3

IV.3 LYMPHOCYTE-MEDIATED MYOTOXICITY: LYMPHOCYTE DOSE RESPONSE STUDIES OF PATIENTS WITH ACTIVE OR INACTIVE INFLAMMATORY MUSCLE DISEASE.

IV.3.1 Introduction

In preliminary experiments of myotoxicity reported in Chapter 2, lymphocytes were incubated with ^{3}H-Carnitine labelled human fetal muscle cultures for 18 hours. Although the assay did distinguish differences in myotoxicity between lymphocytes obtained from patients with active inflammatory muscle disease, and those from a control group with non-inflammatory muscle disease, it was hoped to introduce a shorter term assay for further studies.

In the previous chapter, it was seen that lymphocytes from 5 IMD patients exerted significantly greater myotoxicity in absolute terms, against fetal muscle monolayers, than an equivalent number of lymphocytes from patients with non-inflammatory muscle disease. It was not possible to estimate the numbers of myotubes per culture well in individual experiments and thus obtain an accurate effector to target cell ratio. Consequently, myotoxicity could not be calculated on a per lymphocyte basis by conventional means. However, regression analysis of the percentage myotoxicity by the same lymphocyte population at different concentrations would determine whether myotoxicity by

lymphocytes from individual patients was dose related with respect to numbers of lymphocytes added.

In order to allow comparison of results from individual patients on different occasions, and also to investigate myotoxic dose-response relationships, myotoxicity would be better calculated from an estimate directly related to muscle cell killing, and for which limits of variation could be determined. It was hoped that the introduction of a short-term myotoxicity test would allow calculation of myotoxicity in this way, and thus overcome both the technical and statistical limitations involved in 18 hour assays. A 7 hour incubation period was decided upon, in preference to the conventional 4 hour assay usually employed in ^{51}Cr based short term cyototoxicity assays. The introduction of a 7 hour myotoxicity test would have the following advantages:

1) It would reduce the non-specific loss of ^{3}H-C from cultures of HFM and sensitivity of estimates of myotoxicity might increase, and was on the plateau of ^{3}H-C loss from pre-labelled muscle cultures (Chapter II.6).

2) Seven hours is insufficient time for precursor lymphocytes present in peripheral blood lymphocyte populations to be induced to cytotoxic cells, by differences in major histocompatibility complex antigens between monolayer cultures and added lymphocytes (Svedmyr, Wigzell and Jondal, 1974). Consequently, it might be possible to employ culture medium alone as a control treatment for non-specific loss and thus removed the need for a population of lymphocytes from normal

individuals.

This method for calculation of myotoxicity would allow direct comparison of values obtained within each assay, as well as between assays performed on different occasions, in relation to non-specific loss of ^{3}H-C.

However, as in the 18 hour assay, the contribution of spontaneous cytotoxicity to measurements of <u>in vitro</u> myotoxicity cannot be determined. The formula for estimation of myotoxicity in 7 hour assays would become:

$$\% \ ^{3}\text{H-C retention} = \frac{(^{3}\text{H-C retention by monolayers after incubation with lymphocytes})}{(^{3}\text{H-C retention by monolayers after incubation with cultured medium})} \times \frac{100}{1}$$

The values obtained by application of this formula to results of myotoxicity experiments will bear an inverse relationship to the amount of specific muscle cell damage by lymphocytes. Retention of ^{3}H-C by monolayers incubated with medium alone would be taken as the 100% value. In other words, the greater the damage to cultures incubated with lymphocytes, the lower the value for the % ^{3}H-C retention myotoxicity.

Confidence limits could be derived by measuring the standard error of the mean (SEM) of estimates of ^{3}H-C retention by prelabelled HFM mono layers after 18 hours incubation. This was found to lie between 100 ±

15% (II.4.4). However, range of normality has been set at 100 ± 20% because these values lie outside the 95% fiducial limits.

The experiments described in this chapter were primarily undertaken to determine whether there was any correlation between the number of lymphocytes added per well and percentage myotoxicity using a 7 hour assay.

IV.3.2 Experimental design

Serial dilutions of lymphocytes from patients with inflammatory muscle disease, considered to be either clinically active or inactive at the time, were incubated with four replicates of human fetal muscle cultures. After 7 hours incubation at 37°C in a humidified CO_2 incubator, ^{3}H-Carnitine retention by the cultures was measured and expressed as a percentage of that by cultures in the same microtitre plate incubated with culture medium alone. Lymphocyte preparation, culture and labelling of the human fetal muscle cultures and harvesting are described in detail in Section II (Materials and Methods). Myotoxicity is expressed as a function of the reduction in retention of ^{3}H-Carnitine by cultures incubated with lymphocytes compared with that by cultures in the presence of culture medium, over the period of the assay.

IV.3.3 Results

The percentage reduction in ^{3}H-Carnitine retention detected in serial

% ^{3}H-CARNITINE RETENTION

A

ACTIVE — lymphocytes added per well (x 10^{5})

INITIALS	10	5	2.5	1.25
EK	54.7	67.4	74.4	*ND
MM	75.3	78.3	94.0	"
SH	58.1	65.0	77.2	"
SA	47.6	64.4	82.1	"
AW	51.0	55.1	71.0	98.1
ER	43.7	49.9	63.1	86.9

B

INACTIVE — lymphocytes added per well (x10^{5})

INITIALS	10	5	2.5	1.25
ER	90.8	90.1	81.3	93.4
BH	100.9	75.6	86.1	83.8
RH	87.4	93.6	103.9	ND
BE	86.3	99.4	106.5	ND
MC	ND	88.2	87.3	103.6
IP	89.5	95.8	98.4	95.3

*not done

Table IV.3.3.i

The percentage ^{3}H-Carnitine retention by HFM monolayers following 7 hours incubation with different numbers of lymphocytes added per well at the initiation of the experiment. The lymphocyte populations were obtained from patients with either clinically active (A), or inactive (B) inflammatory muscle disease.

A ACTIVE

Patient	slope (a)	y-intercept (b)	x-intercept (-b/a)	R value
EK	-17.7	72	4.1	1.0
MM	-21.4	101.3	4.7	0.69
SH	-23.8	80.6	3.4	0.94
SA	-44.2	90.5	2.0	0.98
AW	-45.4	90.0	1.9	0.83
ER	-47.9	66.0	1.4	0.93

B INACTIVE

Patient	slope (a)	y-intercept (b)	x-intercept (-b/a)	R value
ER	2.6	87.7	-33.9	0.19
BH	5.2	87.5	-19.9	0.18
RH	-23.7	110.0	4.6	0.96
BE	-26.8	113.1	4.2	1.0
MC	-31.0	101.7	3.3	0.75
IP	- 8.3	98.6	11.8	0.86

Table IV.3.3.ii

The parameters derived when the data presented in Table IV.3.3.i) was subjected to straight line regression analysis, by application of the formula $y = ax + b$, where x is the number of lymphocytes added per well, and y is the percentage of ^{3}H-Carnitine retained by HFM monolayers after 7 hours incubation with lymphocytes from patients with clinically active (A) or inactive (B) IMD. R is the correlation coefficient.

A ACTIVE

Initials of patient	Treatment Prednisolone (mg/day)	Activity of disease
EK	20	++
MM	60	+++
SH	nil	+++
SA	nil	++
AW	15	++
ER	nil	+

B INACTIVE

Initials of patient	Treatment Prednisolone (mg/day)	Activity of disease
ER	5	*NA
BH	20	"
RH	2	"
BH	35	"
MC	15	"
IP	12	"

*not applicaable

Table IV.3.3.iii

The drug treatment being administered to patients with either clinically active (A), or inactive(B) IMD, who were studied in this chapter. The relative severity of muscle disease in patients described as being 'active' is also indicated, using the same arbitrary scale for clinically determined disease activity as in Table IV.2.3.

dilutions of lymphocytes taken from 6 patients with clinically active disease and 6 with inactive disease are represented in Table IV.3.3i. Drug treatment being adminstered to individual patients is shown in Table IV.3.3.iii. When ^{3}H-Carnitine retention in cultures incubated with lymphocytes was less than 80% of the control value (cultures incubated with culture medium alone), myotoxicity was regarded as significant. Lymphocytes from all patients in the 'active' group showed significant myotoxicity at both 10×10^5 and 5×10^5 lymphocytes per well and four showed killing at 2.5×10^5 lymphocytes per well. In the 'inactive' disease group, only patient S.H. showed a single significant myotoxicity value, 75.8% at a lymphocyte concentration of 5×10^5 per well.

In order to determine whether the values of percentage reduction in ^{3}H-Carnitine retention at different lymphocyte concentrations fitted a particular curve, the data represented in Table IV.3.3.i were subjected to regression analysis. The formula for a straight line, $y = ax + b$, where y = % ^{3}H-Carnitine retention and x is equal to the number of lymphocytes added per well, was found to give the best fit.

The parameters derived from the application of regression analysis to the results, are given in Table IV.3.3.ii. The values for R, the correlation coefficient, are also included in Table IV.3.3.ii, although there were only three data points for most patients. It can be seen from Table IV.3.3.ii,at the values for the slope (a) of linear regression lines of 'active' patients were all negative. Four of the values for the slopes obtained for the 'inactive' group were also negative. However, the corresponding values for the intersection

points on the y-axis (b) in these 4 patients were generally greater than those calculated for the 'active' group.

IV.3.4 Discussion

These results were thought to imply that a dose response relationship between the number of lymphocytes added per well and percentage ^{3}H-Carnitine retention was present in lymphocyte populations from patients with active disease. The lower values of percentage ^{3}H-C retention as shown by the y-axis intercepts for the 'active' disease patient group, compared with those of the 'inactive' group, suggested that the numbers of lymphocytes required to exert myotoxicity was probably less than that shown by lymphocytes from patients with inactive disease.

The results described in this and the preceding chapter have shown that the lymphocytes from patients with active inflammatory muscle disease were myotoxic to cultures of human fetal muscle. In addition, lymphocyte dose responsiveness was observed in myotoxic assays by lymphocytes from these patients. When interpreting these results, it is important to note that no information about the specificity of the cytotoxicity for targets other than human fetal muscle, nor the identity of the lymphocytes responsible for *in vitro* myotoxicity, has been given. These points will be addressed in subsequent chapters.

However, the observations made were considered to be valuable because of the potential of lymphocyte mediated myotoxicity as measured by the ^{3}H-Carnitine retention assay, as a marker for presence or activity of inflammatory muscle disease.

In order to assess further the possible use of this assay, an *in vitro* measure of disease activity in patients with inflamatory muscle disease, sequential measurements of myotoxicity by lymphocytes from three patients were made during the course of their illness. These studies comprise Chapter 4.

IV

CHAPTER 4

IV.4 LYMPHOCYTE-MEDIATED MYTOTOXICITY: LONGITUDINAL STUDIES OF PATIENTS WITH INFLAMMATORY MUSCLE DISEASE

IV.4.1 Introduction

In chapters IV.2 and 3, it was found that lymphocytes from patients with active inflammatory muscle disease were myotoxic to cultures of HFM, while those from patients with inactive disease, or non-inflammatory muscle disease, were not.

The studies reported in this chapter were undertaken to investigate lymphocyte-mediated myotoxicity sequentially, in individual patients with IMD, in conjugation with their clinical state and drug therapy.

The difficulties of clinical assessment in patients with IMD have been discussed in the Introduction to this thesis. Individual patients vary a great deal in initial symptoms, severity of illness, patterns of relapse and remission, response to therapy and sequelae.

In order to limit variation between clinical assessments as much as possible, it was decided to include only those patients who were under the care of the same clinician throughout the course of their illness. Thus, each patient was his/her own point of reference with respect to relapse or remission and response to therapy as determined by this

clinician at each examination. Unfortunately, because of the rarity of this condition and problems of clinical organisation, only 3 patients were able to be studied sequentially in this manner. All 3 patients were children attending the Muscle Clinic at Hammersmith Hospital.

Clinical criteria of diagnosis and disease activity are those used in the Department of Paediatrics and Neonatal Medicine at Hammersmith Hospital. It is not within the scope of this thesis to justify or discuss the assessment or management of these patients.

The general features in the course of illness and a summary of laboratory and pathological investigations are included for each patient. Another factor which had to be taken into account for these studies was that the frequency and amount of blood taken from each child, for studies of myotoxicity, was limited by consideration for the child's well-being and haematological state. However, seven-hour myotoxicity tests using reduction in ^{3}H-Carnitine retention as a measure of specific muscle cell damage on serial dilutions of lymphocytes from these patients were undertaken whenever practicable.

IV.4.2 Results

The results of the serial studies are given in the following way. For each patient a chart, representing the salient features in the course of illness, together with steroid and other drug treatment over a period of time pertinent to the study, has been compiled. These comprise Figures IV.4.2.1 (Patient A.W.), IV.4.2.2 (Patient C.S.), and IV.4.2.3 (Patient G.H.). The dates on which blood was taken for

myotoxicity tests are indicated by an arrow and each occasion individually numbered in italics. The results of the 7 hour myotoxicity tests for each patient are correspondingly represented in Figures IV.4.2.1A, IV.4.2.2A and IV.4.2.3A. These figures consist of separate individual plots of myotoxicity expressed as percentage ^{3}H-Carnitine retention as a function of lymphocyte concentration per well, corresponding to the appropriate italic numbered experiment indicated in Figures IV.4.2.1, IV.4.2.2 and IV.4.2.3.

The level of significance for <u>in vitro</u> myotoxicity has been previously determined as 100 ± 20% ^{3}H-Carnitine retention in the presence of lymphocytes. The 100% value for ^{3}H-Carnitine retention was that measured for muscle cultures incubated with culture medium alone over the period of the assay (7 hours). The result for each patient will be discussed in conjunction with clinical features and drug therapy, followed by a general discussion based on these serial studies.

<u>Patient A.W. (Female, Date of Birth 17.7.75)</u>

Refers to Figures IV.4.2.1 and IV.4.2.1A (i-v).

This child was first diagnosed as having inflammatory muscle disease in June, 1979, at the age of nearly 4 years.

Onset of symptoms was first noticed around Christmas, 1978, when this child showed a personality change - becoming tired easily, miserable and refusing to play at school, which contrasted with her usual well-

being. She developed a rash on her nose and below her eyes and both legs appeared slightly swollen. In April 1979 she experienced difficulty in walking and getting up from the floor. She was not seen at Hammersmith Hospital until June, 1979, when a diagnosis of polymyositis was made.

Investigations include a right quadriceps muscle biopsy, which showed widespread muscle necrosis, electromyography results indicative of myopathic changes, and a creatine phosphokinase level of 2830 i.u. (normal F < 170 IU). Auto-antibody and complement investigations showed no abnormalities.

She was immediately treated with prednisolone at a dose of 15 mgs per day, which was subsequently raised to 20 mg/day. Following two weeks on this dose of steroids, her weakness appeared to be resolving and the steroids were gradually decreased.

When first studied in August and receiving 10 mg/day prednisolone, her lymphocytes showed no significant myotoxicity in the test (Fig. IV.4.2.1Ai. She continued to improve and the steroid dose was gradually decreased. Her next test was carrid out in early October, when she was receiving 5.5 mg/day prednisolone, and significant lymphocyte dose-related myotoxicity was observed (Fig. IV.4.2.1Aii). However, clinically she appeared to be steadily improving and her parents were told to continue reducing the steroid dose (as they had been doing for the past two weeks). Within a week, the child developed dysphagia and a worsening of her muscle weakness. Steroids were increased to 7.5 mg/day and she rapidly improved. The next test was

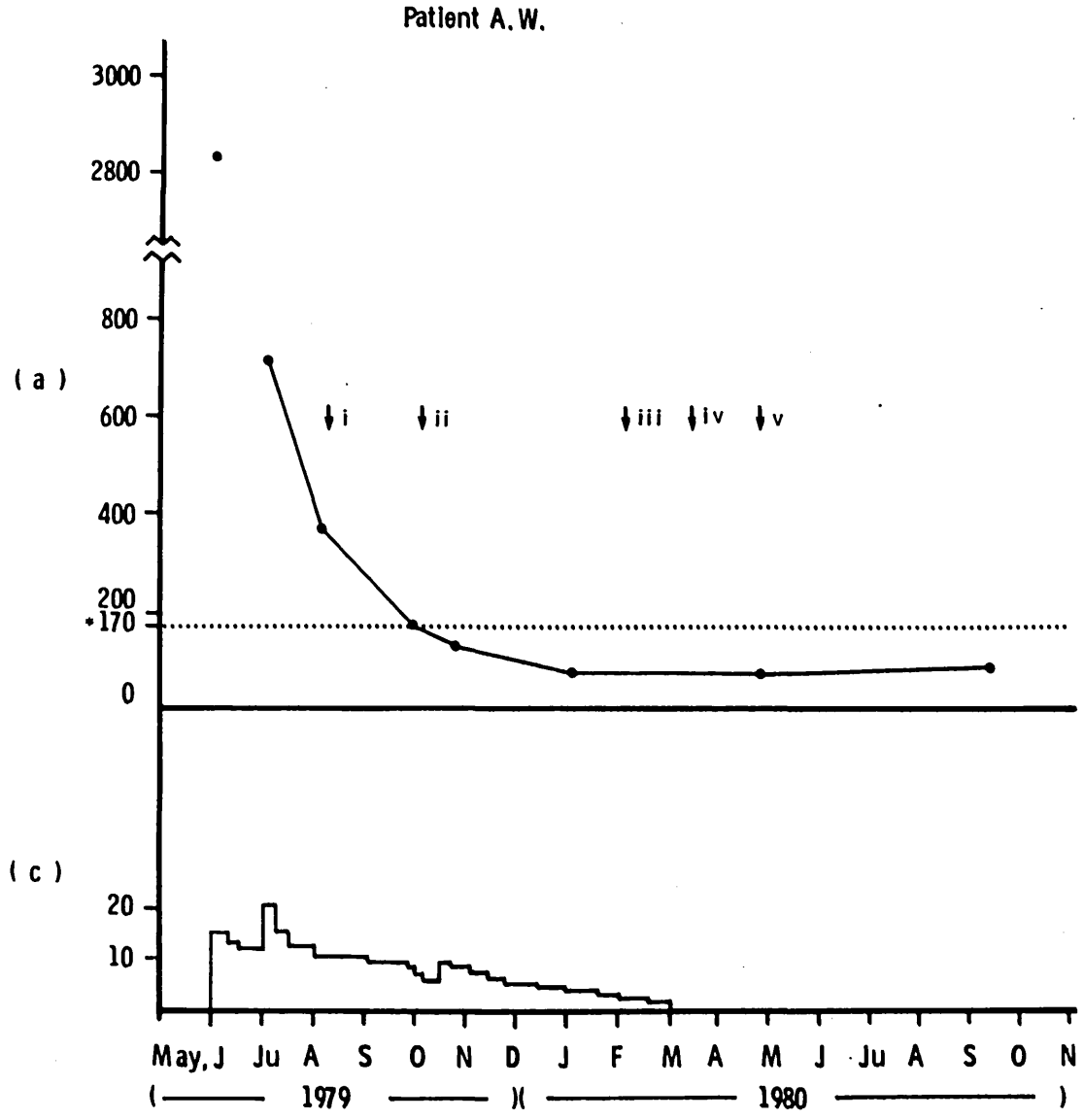


Fig IV.4.2.1

Patient AW. Serum creatine phosphokinase enzyme (CPK) values for this child are given on the (a) scale; the upper limit for normal CPK levels in a female child of this age being denoted by the dotted line. Prednisolone (mg/day) being administered to AW over the course of study is shown on the (b) scale. The occasions when blood was taken for myotoxicity studies is here indicated with an arrow and are numbered sequentially in italics.

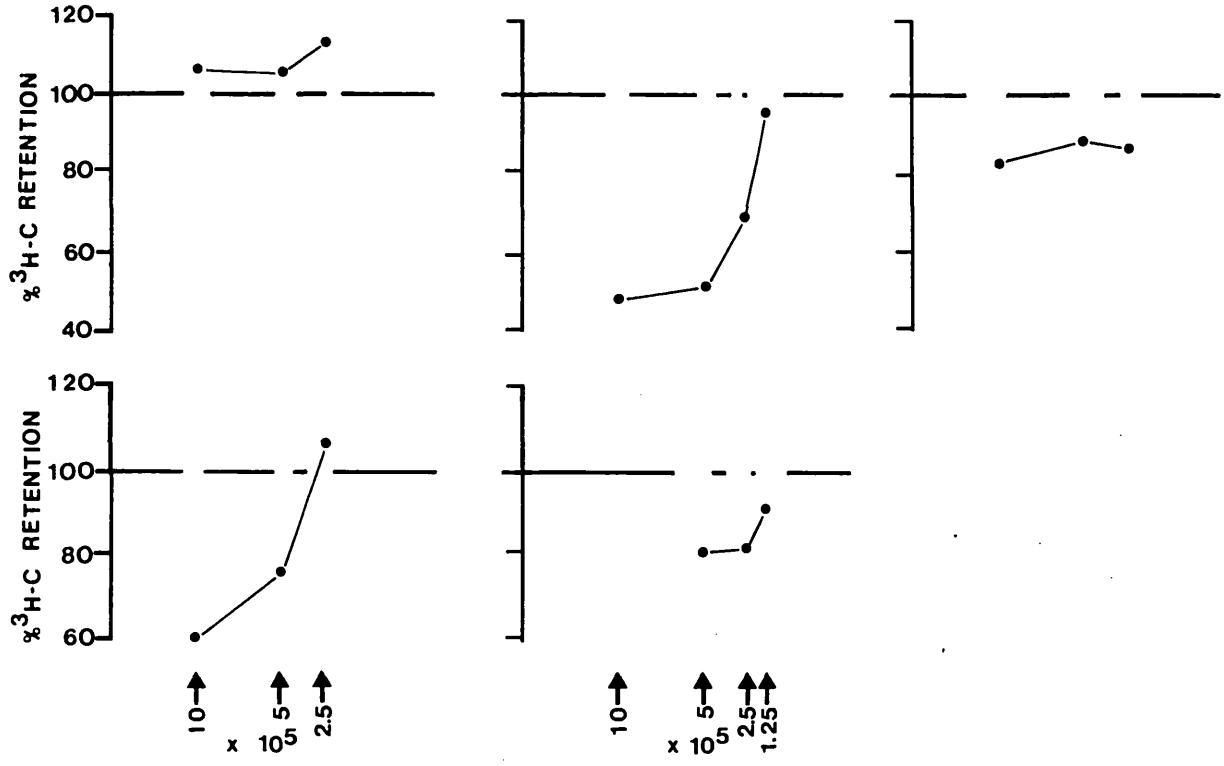


Fig IV.4.2.1A (i-v)

Patient AW. The percentage ^{3}H-Carnitine retention by monolayer cultures of HFM after 7 hours incubation with different concentrations of lymphocytes from AW is shown for the times of study indicated in italics in Fig IV.2.1.

performed in February of the following year, when she appeared well, and the prednisolone dose was down to 1 mg per day. The results of the lymphocyte-mediated myotoxicity test (Fig IV.4.2.1Aiii) were within the limits of variation for the test. Steroid treatment was stopped shortly afterwards. The next test was performed when she attended the hospital in the middle of March, and significant lymphocyte dose-related myotoxicity was found (Fig. IV.4.2.1Aiv). She seemed much improved, although muscle weakness was still apparent. Within days, however, deterioration in her condition was noted by her parents and also when she was subsequently examined at Hammersmith Hospital. It was decided not to resume treatment and she had improved greatly within a month of this episode. Myotoxicity was not in evidence from a test performed at the end of April (Fig. IV.4.2.1Av) and until the present time (1982) there have been no further relapses.

Patient C.S. (Female, Date of Birth 17.7.75)

(Refer to Figures IV.4.2.2 and IV.4.2.2A(i-vi).

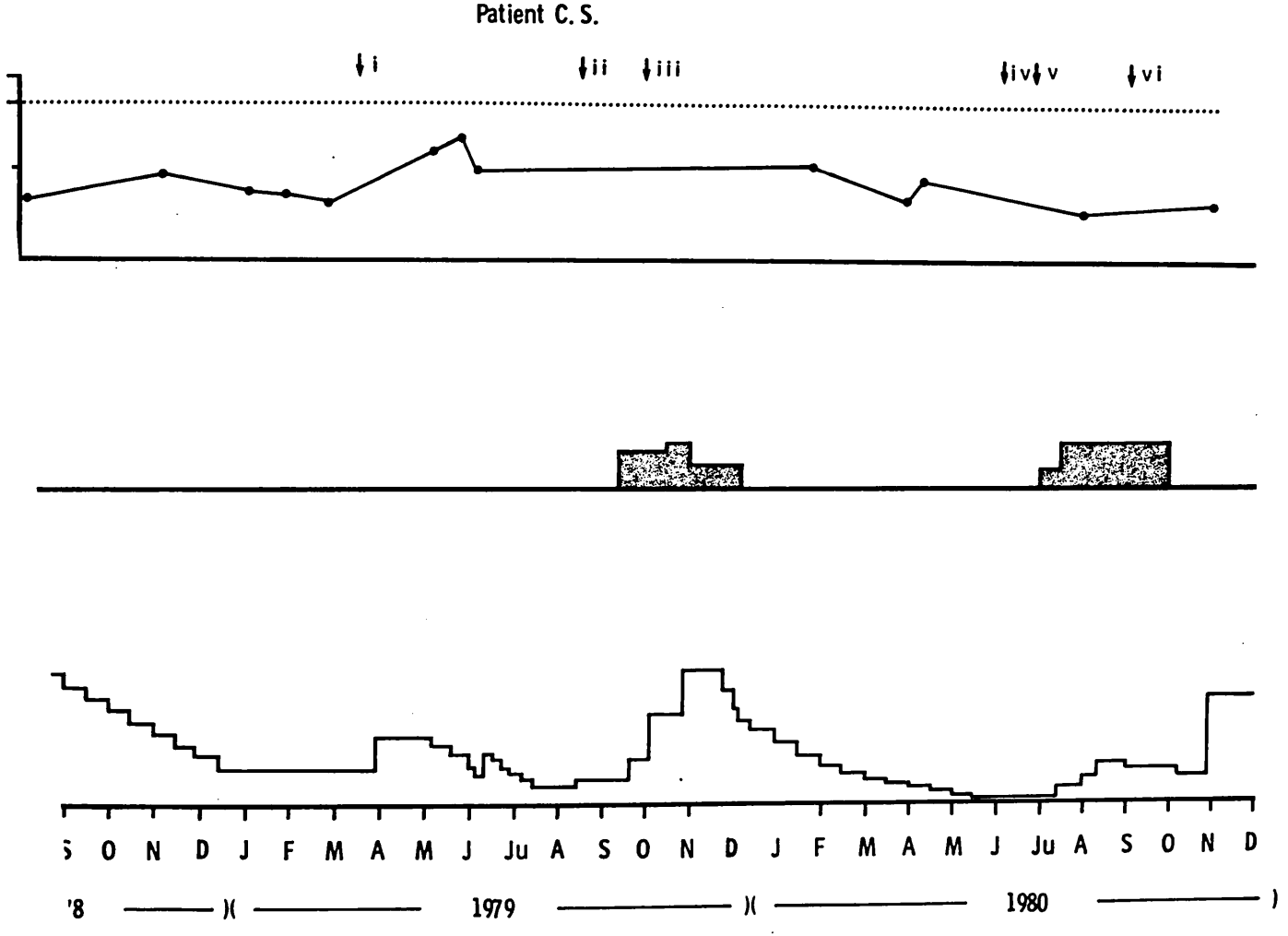
C.S. was two years old and resident in Lisbon, Portugal when she first developed signs of muscle weakness and an erythematous rash on her hands and face. In April 1978, she became increasingly tired and miserable. She was treated with 2 mg prednisolone/day for several weeks at a hospital in Lisbon, which seemed to bring about slight improvement. However, a month later she became increasingly weak and miserable, and her parents brought her to Hammersmith Hospital at the beginning of August 1978. A diagnosis of dermatomyositis was made on clinical, biopsy and EMG grounds. Muscle biopsy showed marked

inflammatory changes and fibre necrosis and electromyography revealed myopathic features. Her CPK, however, was within normal limits and no auto-antibodies or complement abnormality was apparent.

She began immediate treatment with 30 mg/day of prednisolone and showed marked improvement within one week. The dose was gradually reduced to 7.5 mg/day prednisolone in the next five months. Her first myotoxicity test was done in March 1979, when she was still on this dose (Fig IV.4.2.2Ai). It showed marginal myotoxicity by lymphocytes, at the two highest concentrations, per well tested. Her steroid dose was increased shortly after this because of a deterioration in her condition which became markedly worse in May. Following a slight improvement in June, she relapsed again in July. She was seen at this time (in early August, 1979) when receiving 4.5 mg prednisolone per day (Fig IV.4.2Aii). The test showed marked myotoxicity at the highest lymphocyte concentration and this was also evident at the next two serial dilutions of lymphocytes. At this point in her illness, the vasculitic rash on her face and hands worsened and her weakness became more pronounced. Azathioprine, (40 mg/day) was added to her drug regime of 5 mg prednisolone per day, in early September. However, her weakness continued to increase, despite now receiving 10 mg prednisolone/day, and 40 mg/day azathioprine. When tested at the beginning of October (Fig. IV.4.2Aiii) marked myotoxicity was shown by lymphocytes at the two highest concentrations tested. Her steroids were increased to 30 mg prednisolone/day and her weakness became less marked. Although vasculitis was still a problem, azathioprine had to be withdrawn because of toxicity problems. Over the next 6 months, her overall condition gradually improved, and steroids were reduced over

Fig IV.4.2.2

Patient CS. The results for this child are represented in a similar way to those of AW in Fig. IV.4.2.1. However, in this figure the (c) scale shows prednisolone dosage (mg/day) and scale (b) azathioprine being administered (mg/day) in addition.


Patient C. S.
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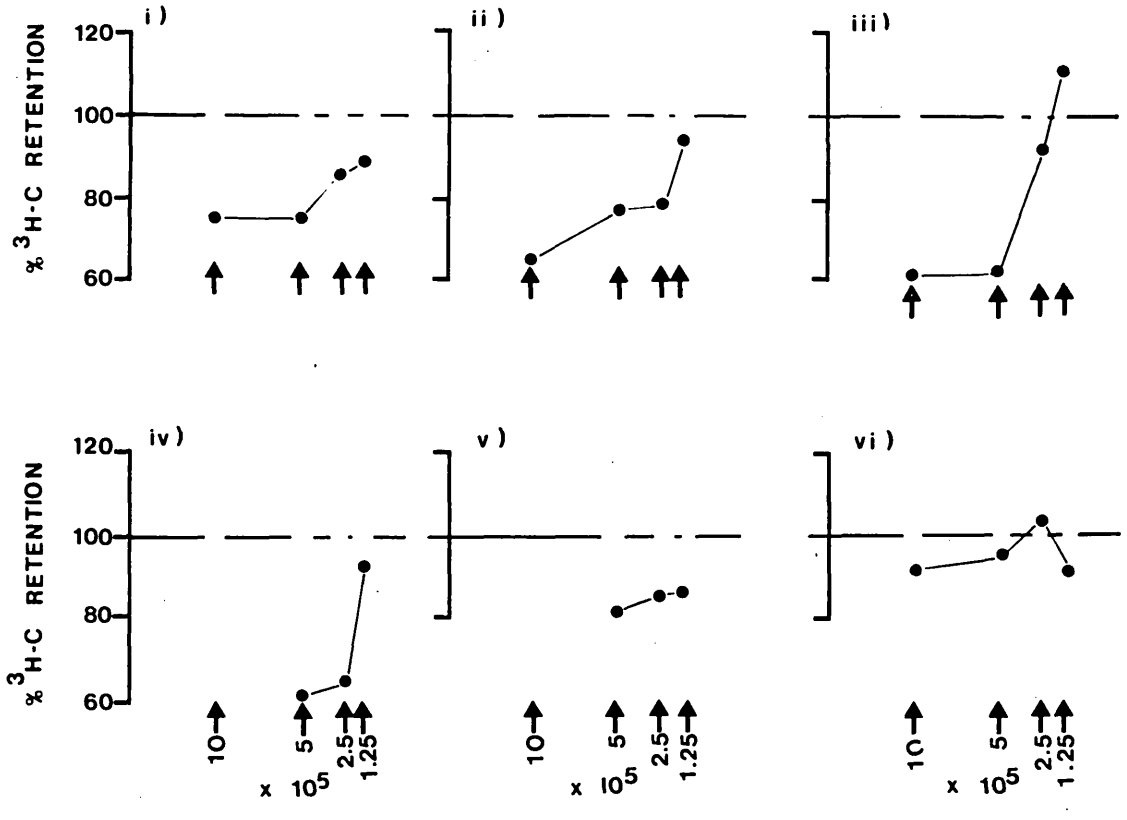


Fig IV.4.2.2A (i-vi)

Patient CS. The results shown here for this child are represented similarly to those in Fig IV.4.2.1A.

this period to 3 mg prednisolone per day, which she was receiving when she was next studied at the beginning of June, 1980. Myotoxicity was marked at the two highest lymphocyte concentrations tested (5 and 2.5 x 10^5 lymphocytes per well) as seen in Fig. IV.4.2Aiv. At this time her condition was beginning to deteriorate, and within a week of the test, muscle weakness was very pronounced. In addition, her vasculitic rash had become more severe and spread over her abdomen. The test performed when vasculitis was very severe (Fig. IV.4.2.2Av) showed no myotoxicity by her lymphocytes at any concentration. Azathioprine was added to increasing doses of steroids shortly after this. The next myotoxicity tests were performed in August 1980 (Fig IV.4.2.2Avi) when she was receiving 50 mg azathioprine plus 10 mg prednisolone per day. This test revealed no evidence of lymphocyte-mediated myotoxicity.

It is noted that the highest lymphocyte concentration of 10 x 10^5 lymphocytes per well, usually employed in the myotoxicity assay, was not tested on the last three occasions when this child was studied. This was because her total white blood cell count and haemoglobin were both low at these times. Since September, 1980, the main features of her disease have been recurrent bacterial infections and vasculitis. She has also developed severe contractures in both arms and it is thus difficult to assess muscle weakness, although the clinical opinion is one of 'burnt out' dermatomyositis, with weakness due to sequelae following active muscle disease.

Patient G.H. (Male, 29.5.68)

Refer to Figure IV.4.2.3 and IV.4.2.3A(i-vi).

Towards the end of 1978, this previously healthy 10 year old boy developed periorbital oedema, and an erythematous rash over his cheeks and eyelids. This was followed by the appearance of a similar rash over his knees, knuckles and elbows. One month later, he started to become progressively weaker, was unable to sit up from lying, and had difficulty swallowing. In December 1978 he was diagnosed as having dermatomyositis, and was started on 40 mg prednisolone per day.

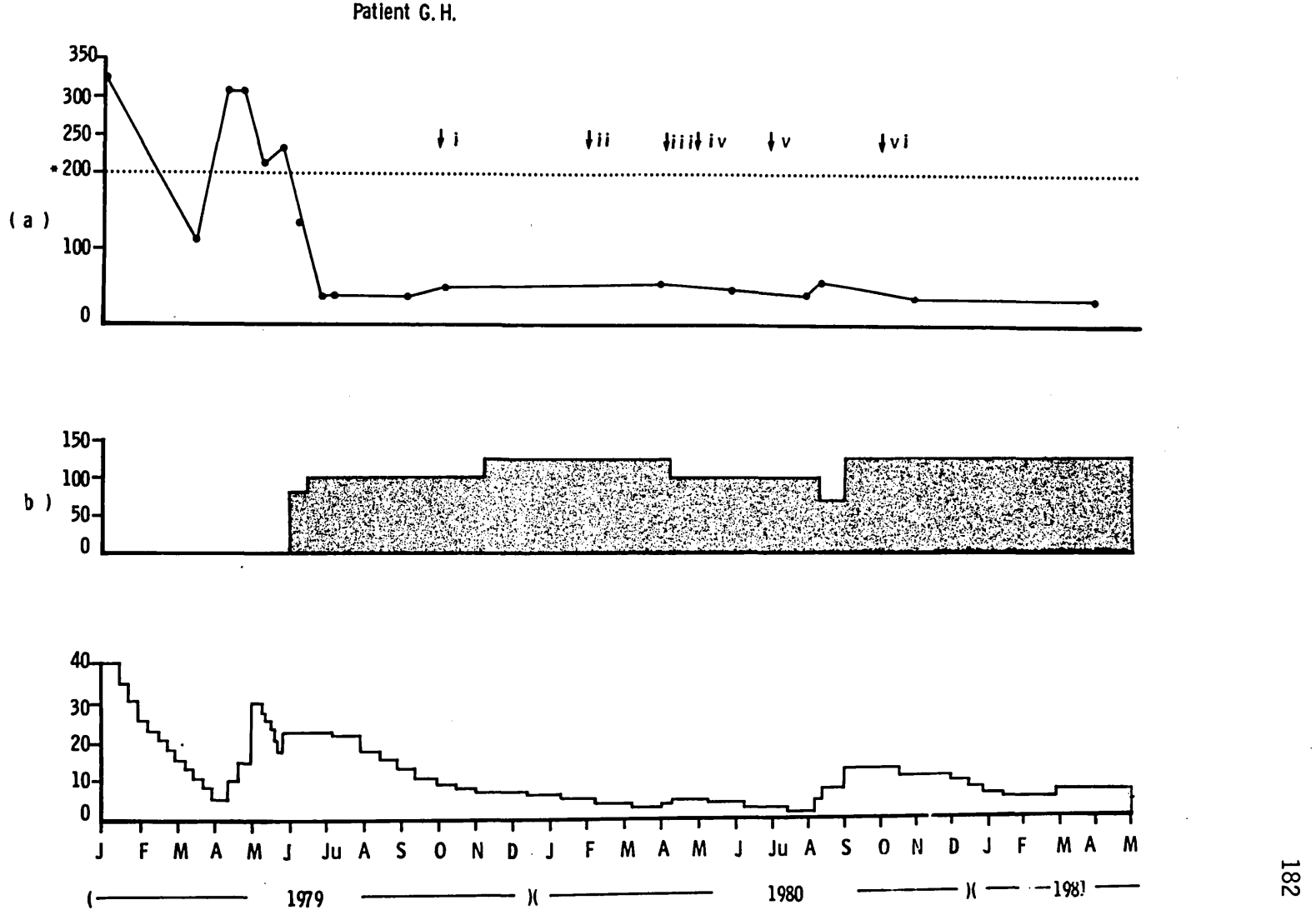
A muscle biopsy performed at this time was reported as being within normal limits and having no evidence of muscle inflammation. In addition, results of electromyography of the right quadriceps muscle at this time were judged to be 'virtually normal'. Creatine phosphokinase levels (CPK) were elevated in December 1978 (Fig IV.4.2.3). Shortly after the onset of illness, however, they decreased to within normal limits by March, following the introduction of steroid therapy. A rise in this enzyme was recorded, in coincidence with reduction in steroids, and relapse in April 1979, but by the end of May that year had again fallen to within the normal range. No other elevations of the CPK enzyme have been recorded since then.

By the end of January (1979) he had greatly improved in strength, although there was no change in the rash. The steroid dose was decreased gradually to 5 mg/day by the middle of April, when he had a relapse. He was unable to get up from the floor, and the periorbital

oedema increased. Despite an increase in prednisolone dose to 30 mg/day, by the middle of May, there was no improvement, and after decreasing the dose to 22.5 mg/day at the end of May, azathioprine (75 mg/day) was added to the drug regime, which was subsequently increased to 100 mg/day two weeks later. When the first myotoxicity test was done at the beginning of October 1979, one year after diagnosis, (Fig. IV.4.3.Ai) it showed very low levels of myotoxicity at the two highest lymphocyte concentrations tested. When next seen in June, 1980, a slight improvement in muscle strength was noted following the recent introduction of azathioprine. The prednisolone dose had been decreased to 10 mg/day. However, one month later, no further improvement was detectable and azathioprine was increased to 125 mg/day. He once again started to improve, and by the end of January 1980 was able to run and climb stairs, still with some difficulty, but these activities had not been possible at all during the previous 12 months. His lymphocytes showed no evidence of myotoxicity at any concentration tested (Fig. IV.4.2.3ii). Prednisolone was decreased to 3 mg/day by the end of March, (1980), but was increased to 6 mg/day during April when he again relapsed. He was again unable to negotiate stairs, and the erythematous rash on his eyelids and knuckles worsened. At this time his total blood count was so low as to cause concern, so azathioprine was decreased to 100 mg/day in the first week of April. Myotoxicity assays performed at the beginning and end of April (Figs IV.4.2.3iii and iv) showed marginally significant myotoxicity at the highest lymphocyte concentration tested only. The test carried out at the end of April, when weakness was pronounced and azathioprine had been decreased, showed low, but detectable myotoxicity at the two highest lymphocyte concentrations tested. He started to improve rapidly during

Fig IV.4.2.3

Patient GH. CPK results and drug treatment in this child are represented in the same manner as for CS.


Patient G.H.
350
300
250
200
100
0
(a)
i
ii
iii
iv
v
vi
150
100
50
0
b)
40
30
20
10
0
J F M A M J Ju A S O N D J F M A M J Ju A S O N D J F M A M
1979
1980
1981

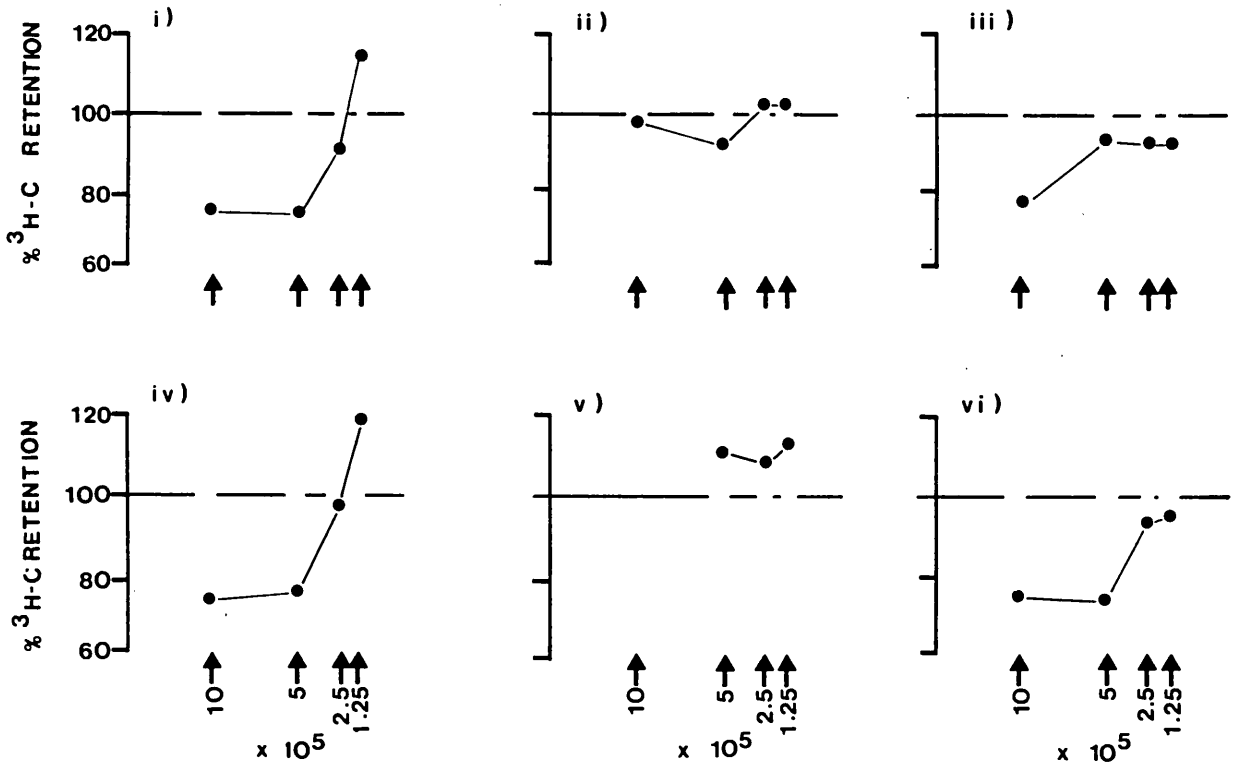


Fig IV.4.2.3A (i-vi)

Patient GH. The result shown here for GH, are represented similarly to those for AW and CS.

May and June and by the beginning of July was on 3 mg/prednisolone/day. The myotoxicity test performed at the end of June (Fig IV.4.2.3.v) showed no detectable myotoxicity at any lymphocyte concentration tested. At the end of July, this child again experienced difficulty climbing stairs, and his muscle function tests showed increasing weakness. Prednisolone was increased to 12.5 mg/day as was azathioprine to 125 mg/day. Blood was taken for the myotoxicity test (not indicated). However, insufficient viable lymphocytes were recovered from 20 ml of blood for this to be done.

The next test was carried out at the end of October, 1980 (Fig IV.4.2.3vi), when slight improvement in muscle strength was noted and the prednisolone dose had been reduced to 10 mg/day. The results showed low levels of myotoxicity at the two highest lymphocyte concentrations tested only. At this stage in his illness, the vasculitic rash which had become more prominent feature of the disease over the last six months, began to ulcerate. At the end of 1980 until May 1981 he was still weak, although able to climb stairs and get up from the floor. However, vasculitis seemed to become increasingly difficult problem to manage. A myotoxicity test carried out in March 1981 showed no cyotoxicity (results not shown).

Routine immunological investigations revealed several abnormalities. Low titre (1/10-1/20) antinuclear antibodies were present in sera taken from this child throughout the course of these studies. In addition, although his total complement titre (CH_{50}) was within normal limits near the onset on illness (June 1979, CH_{50} = 74; normal range 65-135), sequential measurements of his CH_{50} after August to the present time

have consistently shown no detectable activity (CH_{50} < 10). Further investigation has not revealed any specific deficient in complement components C_2 or C_4.

IV.4.3 Discussion

Of the three children studied serially, patient A.W. is the only one at the time of writing who seems to have totally recovered from her illness. The most notable clinical difference between this patient and the other two children is that, apart from a mild, eczema-type rash on her nose and eyes when first seen in June 1979, no other occurrence of skin involvement has been observed. Of the three patients, this child had the most grossly elevated CPK (2830 in June 1979) which fell rapidly after administration of steroids and has been within the normal range since October 1979. Exacerbation in March 1980 did not result in another elevation of the CPK enzyme.

On all occasions when blood was taken for myotoxicity tests, patient A.W. appeared generally well, although with slight muscle weakness. However, myotoxicity was significant on two of these occasions (Fig. IV.4.2.1Aii and iv). Within several days of each of these positive tests for myotoxicity the child relapsed with increased muscle weakness, and on one occasion, dysphagia. Also of note in the course of A.W.'s disease was her rapid initial response to steroid therapy, in June 1979. Following relapse in October 1979, an increase from 4.5 mg to 7.5 mg prednisolone/day was sufficient to control her symptoms. Her second relapse in March 1980 was not treated with steroid therapy, and she improved spontaneously within a month. Her periods of remission

showed correspondingly negative findings in the myotoxicity test (Figs. IV.4.2.1i and v).

Patient C.S. was not seen at Hammersmith Hospital until 18 months after the onset of her disease in 1977. Her disease has proved difficult to control with steroid therapy alone, and since December 1978 she has suffered increasingly from chronic vasculitis and repeated ulceration. Myotoxicity by lymphocytes from this child was significant on four occasions.

The first test, in March 1979 (Fig. IV.4.2.2Ai), showed marginal myotoxicity at the highest lymphocyte concentration tested and her condition was beginning to deteriorate at this time. She had initially responded well to 30 mg/prednisolone/day beginning the previous year, with decreasing doses to 7.5 mg per day at the time of this test. By May 1979 she had pronounced muscle weakness, followed by a brief recovery in June and then another, even more marked, relapse began in July. In early August, myotoxicity was significant and in October when her vasculitis and muscle weakness was severe, myotoxicity had increased at the two highest lymphocyte concentrations per well compared with the August test.

Her next myotoxicity test was performed in June 1980 and significant myotoxicity recorded at both 5 and 2.5 x 10^5 lymphocytes/well. At this time, her vasculitis was beginning to undergo another exacerbation. However, a test undertaken two weeks following this positive result showed no significant myotoxicity, although again, no measurement for lymphocytes at 10 x 10^5 /well could be included. The final two

myotoxicity tests, in August and September 1980, were performed when the patient was receiving 50 mg/azathioprine/day and 10 mg/prednisolone/day.

Although myotoxicity was detected on several occasions during the course of G.H.'s illness (Fig. IV.4.2.3Ai,iii,iv and vi) at no time was this activity marked. Percentage ^{3}H-C-retention of 75% was the highest level of myotoxicity recorded. However, these readings did seem to correlate with exacerbation of disease. It must also be noted that all tests of myotoxicity were performed when azathioprine at a dose of at least 100 mg/day was being administered to this child in addition to low-dose steroids. Whether the low levels of myotoxicity recorded were due to the effects of these drugs, or because there was no myotoxicity to be detected, cannot be determined. However, it should be noted that lymphocytes from C.S. did show significant levels of myotoxicity when she was receiving 40 mg azathioprine plus 10 mg/prednisolone/day, but not when she was on higher doses of this drug.

G.H. had several other different features of this disease when compared with the other two children. He was the only one to have a positive finding for auto-antibodies (ANA positive at low titre) and some evidence of complement consumption. It is also of interest that he had a normal muscle biopsy and EMG. As in patient C.S., the effect of azathioprine on the test was not investigated.

IV.4.5 Conclusions

The application of the ^{3}H-Carnitine retention based lymphocyte-mediated myotoxicity assay to these 3 children studied during the course of their illness seemed useful as an *in vitro* correlate of disease activity, particularly in the patients A.W. and C.S. In addition, the results of several tests predicted relapses in the absence of positive clinical and laboratory symptoms indicative of increasing disease activity, although the children were all receiving corticosteroids on these occasions. However, when vasculitis became a dominant feature of the disease, or azathioprine was being administered, the results of the test were negative with respect to presence or activity of disease. Whether this was because the *in vitro* test results were reflecting different stages of the disease, or the effect of azathioprine on lymphocyte function could not be determined from these experiments.

IV

CHAPTER 5

IV.5 LYMPHOCYTE-MEDIATED MYOTOXICITY: STUDIES ON PATIENTS WITH OTHER CONNECTIVE TISSUE DISEASES

IV.5.1 Introduction

Studies of lymphocyte-mediated myotoxicity in patients with inflammatory muscle disease have so far indicated that their lymphocytes were myotoxic to cultures of human fetal muscle when muscle disease was active. Quantitation of myotoxicity in individual patients also revealed that this *in vitro* killing was correlated with lymphocyte concentration.

The specificity of the assay for lymphocytes from patients with inflammatory muscle disease has only been investigated with respect to a control group of patients with non-inflammatory muscle disease (Chapter IV.2). These patients had congenital muscle disease with no evidence of an immunological aetiology or pathogenesis.

In order to further investigate the specificity of *in vitro* myotoxicity for IMD it was decided to include a control group of patients with other forms of connective tissue disease. The group of patients chosen for study in this chapter consist of 9 patients with systemic lupus erythematosus (SLE) and 5 with scleroderma, plus one patient with Sjogren's syndrome, who is included in the scleroderma group. These are

all conditions in which immunological abnormalities feature strongly in the results of investigations. A myositis can occur in these groups of patients (Bohan et al, 1977). However, clinical assessment of patients was not known to the author at time of study, and were only revealed in retrospect, at the end of the trial. All SLE patients were attending an out-patient clinic at University College Hospital and those with scleroderma and Sjögren's at Dr. C. Black's outpatient clinic at Hammersmith Hospital. Brief summaries of all patients studied, including drug therapy and evidence or otherwise of muscle involvement, are given in Table IV.1.6ii.

Myotoxicity by lymphocytes from these two patient groups was assessed by adding serial dilutions of lymphocytes to human fetal muscle cultures pre-labelled with ^{3}H-Carnitine as previously described (Section II).

IV.5.2 Results

The results of myotoxicity assays performed on peripheral blood lymphocytes from a group of 9 patients with systemic lupus erythematosis are represented in Fig. IV.5.2i. Two of these patients (A.M. and M.C.) were studied on two occasions, approximately 6 weeks apart (Table IV.5.2). Both patients were also revealed to have a history of muscle involvement in conjunction with their SLE (Table IV.1.6ii). Patient A.M. was the only one with evidence of lymphocyte mediated myotoxicity in the *in vitro* assay. On this occasion, her CPK was elevated (1,137 international units (IU), normal < 130). When re-tested 6 weeks later, her CPK had decreased to 251 IU and *in vitro*

Initials of patient	% ^{3}H-CARNITINE RETENTION Lymphocytes added per well (x 10^5) 10	5	2.5	Treatment *Pred. mg/day	Other
AM	52	78	97	30	-
"	97	86	102	25	-
MC	89	99	113	25	*Aza.
"	104	99	94	9	Aza.
DH	94	120	117	nil	-
BB	77	78	108	nil	*NSAID
DJ	84	88	95	nil	NSAID
FC	101	95	98	8	-
MM	105	78	100	25	-
PP	100	89	93	20	-
PB	104	101	99	25	-

*

Pred: Prednisolone

Aza.: Azathioprine (at least 100 mg/day)

NSAID: Non-steroidal anti-inflammatory drugs.

Table IV.5.2.i

The results of lymphocyte-mediated myotoxicity tests, showing percentage ^{3}H-Carnitine retention by lymphocytes from 9 patients with SLE. The drug treatment which these patients were receiving at the time of each test are also given. Patients AM and MC, were studied on two occasions. In the case of AM, 8 weeks separated the two measurements, and MC, 5 weeks. The results of these myotoxicity tests are also presented graphically in Fig IV.5.2.i.

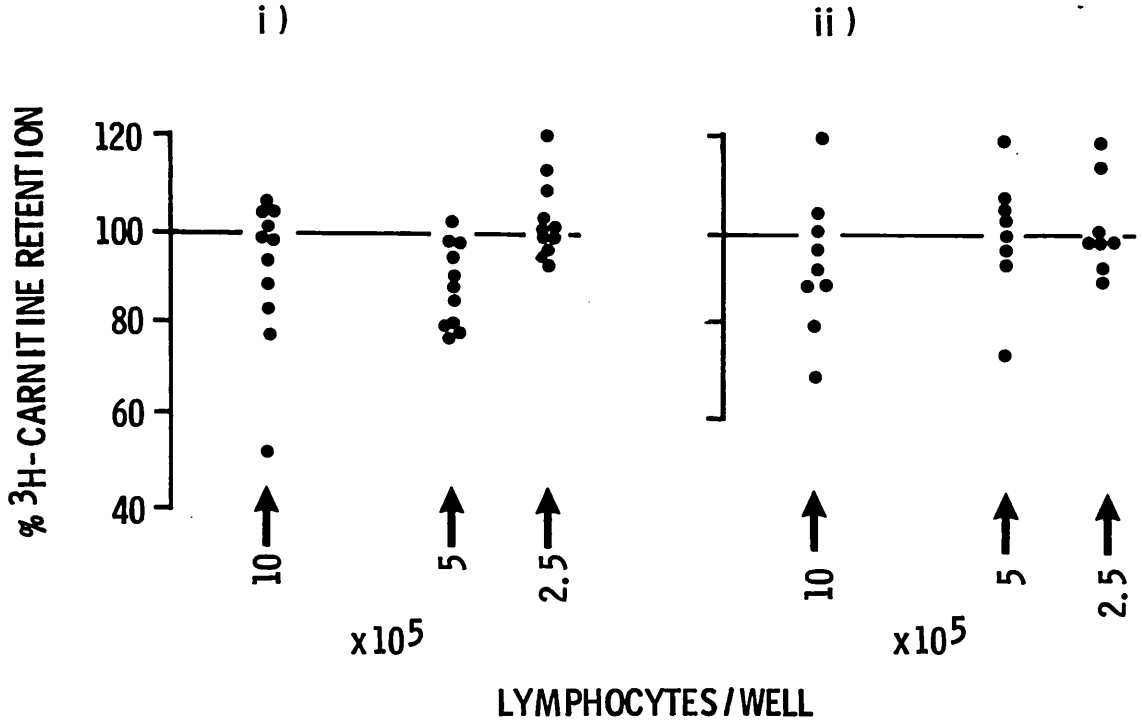


Fig IV.5.2 (i and ii).

The results of lymphoctye-mediated myotoxicity tests, showing percentage ^{3}H-Carnitine retention on the y-axis, and lymphocytes added per well on the x- axis, form studies of 9 patients with SLE (i), and 6 with scleroderma (ii). Tables IV.5.2.i and ii give the values measured for each individual patient and drug treatment. Clinical details on all the patients studied are included in Table IV.1.6.ii.

Initials of patient	% ^{3}H-CARNITINE RETENTION Lymphocytes added per well (x 10^{5}) 10	5	2.5	Treatment *Pred. mg/day	Other
HH	88	107	97	nil	Colchicine
NP	88	99	114	"	D-Pene-illamine
MB	104	106	ND	"	-
"	69	75	90	15	-
CM	79	74	92	nil	-
"	120	119	120	"	-
JW	91	93	99	nil	-
"	95	96	98	"	-
CT	100	102	100	"	-

*Pred: Prednisolone

Table IV.5.2.ii

The results of lymphocyte-mediated myotoxocity tests, showing percentage ^{3}H-Carnitine retention by lymphocytes from 6 patients with scleroderma. Patients MB, CM and JW were studied on two separate occasions, each 4 weeks apart. The results of these myotoxicity tests are also presented graphically in Fig IV.5.2.ii. Also included in this table is the drug treatment which the patients were receiving when tested.

myotoxicity was not shown by her lymphocytes at any concentration. Lymphocytes from the other patient with a diagnosis of polymyositis in association with her SLE (Patient M.C.), did not show any myotoxic activity against cultures of human fetal muscle on either occasion of testing. However, her muscle disease was regarded as clinically inactive on both these occasions (CPK < 5, normal < 130), although her SLE was regarded as moderately active at these times, she was also receiving 100 mg azathioprine. The last episode with clinical and laboratory evidence of muscle involvement had been 6-8 months prior to the time of study.

In the second group of patients, (Table IV.5.2ii and Fig. IV.5.2ii), lymphocytes from one of the patients (M.B.) showed significant myotoxicity in the assay at the two highest lymphocyte concentrations per well (10 and 5 x 10^5). This patient was tested on two occasions, approximately one month apart and her lymphocytes had not shown any acitvity in the assay on the first occasion of testing. However, when her lymphocytes did show significant *in vitro* myotoxicity, the serum CPK was found to be elevated, in contrast to the previous occasion.

IV.5.3 Discussion

From these studies of patients with other connective tissue diseases, it can be seen that an *in vitro* measure of lymphocyte mediated myotoxicity may perhaps be useful in deciding whether muscle involvement is present and active in the patient. This may prove clinically important, because similar symptoms can be caused by factors such as steroid therapy, and the myalgia and weakness often

accompanying active SLE, which may or may not indicate the presence of a myositis (Askari, Vignos and Mushowitz, 1976).

IV

CHAPTER 6

IV.6 LYMPHOCYTE MEDIATED MYOTOXICITY: STUDIES ON NORMAL CONTROL SUBJECTS

IV.6.1 Introduction

Experiments described in Chapters 2-5 inclusive have shown that peripheral blood lymphocytes (PBL) from patients with IMD were capable of damaging cultures of human fetal muscle, when active inflammatory muscle disease was present. Lymphocytes from patients with active connective tissue diseases did not exhibit detectable *in vitro* myotoxicity unless a myositis, as shown by a raised CPK or clinical criteria, was present in association with their disease.

In this chapter, peripheral blood lymphocytes from 18 normal individuals were studied for their myotoxic ability *in vitro*.

IV.6.2 Experimental design

Seven hour myotoxicity assays based on the reduction in retention of ^{3}H-Carnitine of previously labelled human fetal muscle cell monolayers were performed as previously described (Section II). Peripheral blood lymphocytes populations from two of the subjects were tested on four separate occasions and two on two occasions, approximately 3 weeks apart. The other 14 individuals were tested once

only. The mean age of these subjects was 29 years and 6 males and 8 females comprised the group. Estimates of percentage reduction in ^{3}H-Carnitine retention were estimated using 10, 5 and 2.5 x 10^{5} effector lymphocytes per replicate well.

IV.6.3 Results

Figure IV.6.3.i represents the results of myotoxicity tests performed on the 4 individuals tested on more than one occasion. It can be seen than on none of the 4 occasions on which lymphocytes from either subjects A or B were studied was significant myotoxicity observed. However, lymphocytes from subjects C and D each showed significant myotoxicity on at least one of the 2 occasions when their lymphocytes were studied, at the two highest lymphocyte concentrations tested.

In Figure IV.6.3.ii, four of the 14 control subjects each tested on one occasion only, were shown to have lymphocytes capable of exerting significant myotoxicity against human fetal muscle monolayers.

When questioned about their health at the time these assays were performed, subjects C and D admitted to having the symptoms of a mild upper respiratory tract infection when significant *in vitro* myotoxicity was shown by their lymphocytes. Two of the 4 subjects (represented in Fig IV.6.3.ii) whose lymphocytes were also significantly myotoxic in the *in vitro* assay, also admitted to having the symptoms of a mild upper respiratory tract infection.

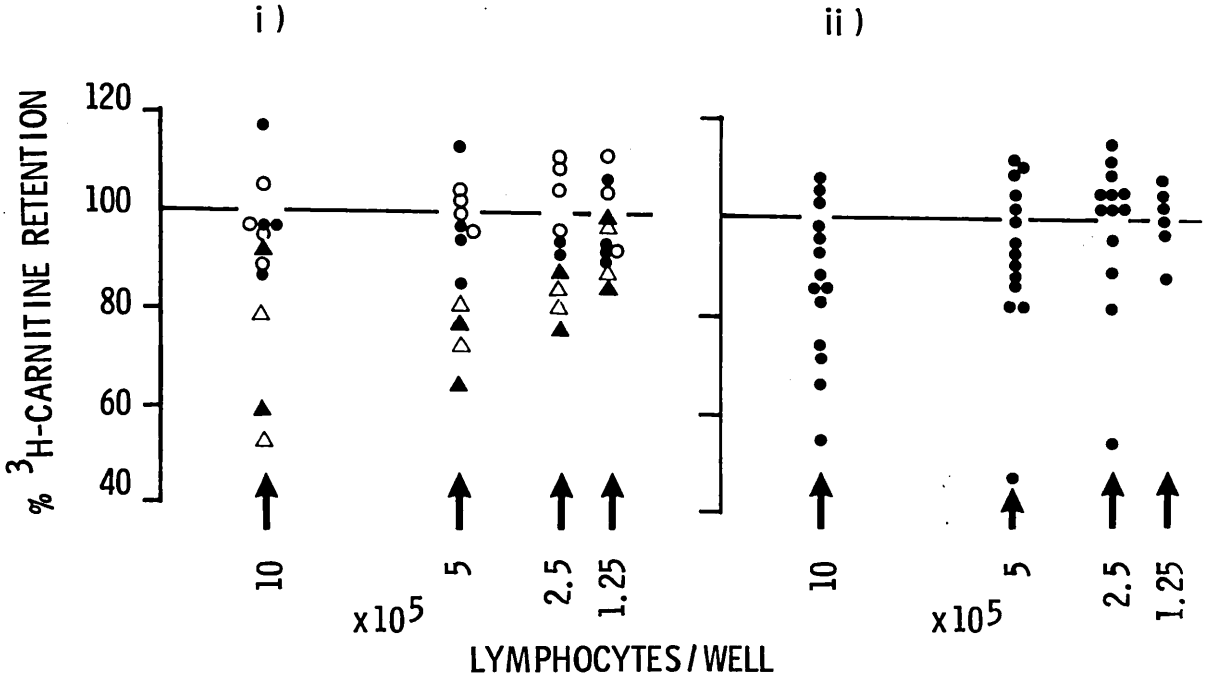


Fig IV.6.3 (i and ii)

The percentage ^{3}H-Carnitine retained by HFM in myotoxicity tests when effector lymphocyte populations from 4 normal individuals were tested on more than one occasion (i), and from 14 normal individuals tested on one occasion only (ii).

IV.6.4 Discussion

The results of myotoxicity experiments have been discussed only in relation to the presence and activity of inflammatory muscle disease in patients, compared with disease control groups. In this chapter, it was shown that lymphocytes from normal individuals were also capable of cytotoxic responses against cultured fetal muscle, especially if subjects were suffering from a mild viral illness. The identity of the effector cell mediating myotoxicity in IMD patients has not been established. However, spontaneous lymphocyte mediated cytotoxic responses, against normal allogeneic cultured human fetal cells other than skeletal muscle, have been described (Herberman and Holden, 1978), using freshly isolated human peripheral blood lymphocyte populations as effector cells. This type of cytoxicity occurs in the absence of anti-target cell antibody and which is not restricted with respect to individual target specificities or antigen specific activation, by products of the major histocompatibility complex (Stern et al, 1980), has been called spontaneous or natural cytoxicity. It is mediated by a heterogeneous population of lymphocytes which are collectively referred to as Natural Killer (NK) cells. The number and efficacy of NK mediated cytotoxicity varies greatly between individuals (Timonen et al, 1979). The final common pathway of their activation *in vivo* and *in vitro* appears to be through their response to and ability to produce interferons (Minato et al, 1980; Timonen et al, 1980). Interferons readily augment the cytotoxic ability of the NK cell population against susceptible targets (Silva, Bonavida and Targan, 1980) and the recruitment of precursor lymphocytes to cytolytically active cells (Saksela, Timonen and Cantell, 1979). In human subjects, serum

interferon levels and NK activity have been shown to increase during virus infections or following the adminstration on interferon inducing agents (Herberman and Holden, 1978; Welsh, 1981). In this chapter, 4/6 normal individuals whose peripheral blood lymphocytes were significantly myotoxic to human fetal muscle cultures had the symptoms of a mild viral illness. It seemed possible that the in vitro myotoxicity shown by lymphocytes from these subjects against fetal muscle targets may reflect the interferon induced activation of NK cells during their illness, as has previously been described using cultured target cells other than muscle cells (Santoli, Trinchieri and Koprowski, 1978). These results also raised the possiblity that the increased in vitro myotoxicity shown by lymphocytes from inflammatory muscle disease patients in the active phase of their disease may be reflecting a general activation of the immune system, including the lymphocyte population which mediates spontaneous cytotoxicity. The possible contribution of spontaneous NK cell mediated cytotoxicity to in vitro measurements of myotoxicity and the target cell specificity of cytotoxic responses by PBL's from inflammatory muscle disease patients and normal controls will be examined in the following chapter.

IV

CHAPTER 7

IV.7 NATURAL CYTOTOXICITY STUDIES

IV.7.1 Introduction

In previous chapters, significant *in vitro* myotoxicity by peripheral blood lymphocyte populations from the patient groups studied was found to be associated with clinically active inflammatory muscle disease. PBL populations from normal individuals could also show significant levels of cyotoxicity against cultured human fetal muscle, especially when the individuals were suffering from a mild viral illness. Spontaneously cytotoxic lymphocytes, known as Natural Killer (NK) cells have the ability to kill a wide range of cultured malignant cells as well as non-transformed, normal fetal, cell targets in the absence of known or deliberate sensitization. Cytotoxicity mediated by NK cells against target cells, has limited selectivity, depending on the susceptibility of target cells to lysis and the cytotoxic efficiency of NK cells which can be enhanced by exposure *in vivo* or *in vitro* to interferon or interferon inducing agents (Casali, Trinchieri and Oldstone, 1983). This results in a general increase in NK mediated cytolytic ability against all susceptible target cells, and renders target cells usually resistent to NK cytolysis, sensitive (Einhorn, Blomgren and Strander, 1978).

Increased *in vitro* myotoxicity by PBL populations from normal

individuals was shown when the interferon inducing stimulus, in the form of a viral infection, was present in the individual tested. This observation, together with the known ability of other fetal-derived tissues to be lysed by NK cells (Timonen and Saksela, 1977), encouraged investigation into possible contribution of NK mediated cytotoxicity to measures of in vitro myotoxicity. It was also thought possible that increased myotoxic activity by PBL populations from IMD patients may reflect a non-specific increase in the spontaneous cytotoxic ability of lymphocytes from these patients during disease exacerbation. Experiments reported in previous chapters have not included studies of the target cell specificity for in vitro lymphocyte mediated cytotoxicity by PBL populations from patients or normal individuals.

Investigation of NK activity of lymphocyte populations usually relies on evaluation of their functional ability to kill susceptible targets in short term 51-Chromium (^{51}Cr) release cytotoxicity assays (Jondal and Pross, 1975). In order to investigate the possible contribution of natural or spontaneous cytotoxicity to measures of in vitro lymphocyte mediated myotoxicity, it was decided to compare the cytotoxic ability of peripheral blood lymphocytes from IMD patients and normal individuals against the convential NK target, the K562 cell line (West et al, 1977), human fetal skin fibroblasts and human fetal muscle. Human fetal skin fibroblasts are minimally susceptible to NK mediated killing (Timonen and Saksela, 1977) In addition, the cytotoxic ability of patient and control PBL populations will be compared with that mediated by peripheral blood lymphocytes depleted of the population mediating the majority of conventional NK activity, that is, the

population of non-B cells bearing receptors for the Fc component on immunoglobulin G, (FcγR bearing cells), (Abo, Cooper and Balch, 1982).

IV.7.2 Experimental design

Although all the experimental procedures used in this chapter have been described in the Materials and Methods section (Section II), it was thought necessary to reiterate some aspects of the methods used in the context of the following experimental results, because of the way in which these results will be presented.

a) Target cells: In experiments where K562 cells or human fetal fibroblasts (HFF) were used as target cells for in vitro lymphocyte mediated cytotoxicity, cell lysis was measured by the calculation of percentage specific ^{51}Cr release into the supernatant from pre-labelled target cells. Cytotoxicity against the K562 cell line was measured in conventional short term killer cell assays of 4 hours duration. When human fetal skin fibroblasts were used as targets, the effector lymphocyte population and ^{51}Cr labelled HFF cells were incubated together for 7 hours, as in myotoxicity experiments.

In experiments where myotoxicity and cytotoxicity against HFF cells were compared using the same effector lymphocyte populations, the HFF and HFM cultures were derived from the same fetus. As in previous experiments, only fetuses of similar gestational age, as measured by fetal foot size, were used as tissue sources in these studies.

b) Effector lymphocyte populations: Peripheral blood lymphocytes

depleted of adherent cells, were prepared as for myotoxicity tests. The effector to target cell (E/T) ratios used when measuring cytotoxicity against K562 and HFF were 100, 50 and 25:1 wherever possible. Unfortunately, insufficient lymphocytes were recovered from samples obtained from patients with IMD, particularly in the case of children, for testing at all three E/T ratios, on many occasions.

In myotoxicity experiments, E/T ratios are not determined because the numbers of myotubes per microtitre plate well vary with the fetal source, although there is little variation between cultures derived from the same fetus. In the following experiments, where myotoxicity and cytotoxicity against K562 or HFF cells were compared using the same effector cell population, the number of lymphocytes added per replicate well were used, instead of E/T ratios. In each experiment, ^{51}Cr labelled K562 or HFF cells were used as targets at a concentration of 1 x 10^4 cells per replicate well.

c) Depletion of lymphocytes with receptors for Fc_γ from peripheral blood populations: Although the population of peripheral blood lymphocytes which express most activity in conventional natural or spontaneous cytotoxicity assays can be purified on the basis of density compared with other lymphocytes (Timonen et al, 1979), the numbers obtained by this method would not be sufficient for the following studies. Therefore, it was decided to deplete peripheral blood lymphocyte populations of those cells which have been shown to express the greatest proportion of NK activity, and compare the cytotoxic ability of populations, before and following depletion. Antisera against a specific surface marker present on the surface of all

conventional NK cells were not available at the time these studies were performed. However, it was known that most peripheral blood NK cell activity could be ablated by removing lymphocytes expressing receptors for the Fc moiety of immunoglobulin-G (IgG). Lymphocytes bearing Fc receptors for IgG (Fcγ R bearing lymphocytes) were depleted from PBL population by means of a solid phase immunosorbent technique (II.3). Interestingly, the recent development of a monoclonal antibody which appears to recognise at least 85% of human peripheral blood NK cells (B 73.1) by Perussia et al (1983) reacts with the Fcγ receptor moiety on the cell surface.

d) Representation of cytotoxicity results: Although the calculation of lytic units per 10^7 lymphocytes, by regression analysis, is the most accurate way of representing the cytotoxic efficiency of effector lymphocyte populations, this was unable to be done in all experiments because often only two data points per sample were available. Therefore, the results of cytotoxicity experiments where ^{51}Cr labelled cells were used as targets, are expressed as percentage specific ^{51}Cr release at a particular E/T ratio or at the number of lymphocytes added to 1×10^4 cells.

IV.7.3 Results

IV.7.3.1 Relative sensitivity of human fetal muscle and fibroblasts to lymphocyte mediated cytotoxicity

i) Experimental proceedure and results: In these experiments, the relative sensitivity of HFF and HFM cultures to cytotoxicity by the

same effector lymphocyte populations was measured. The target cells were ^{51}Cr-labelled HFF, at a concentration of 1 x 10^4 cells per well, and confluent monolayers of ^{3}H-Carnitine labelled HFM derived from the same fetal source as the fibroblasts in each experiment. The effector cells were peripheral blood lymphocytes from 11 normal individuals, 3 of whom were tested twice at 6 week intervals, or PBL from 8 patients with IMD, 2 of whom were tested on two separate occasions (Fig IV.7.3.i and ii). Patients with active disease at the time of testing are depicted by as asterisk in Fig. IV.7.3.1ii. Lymphocytes from each group of subjects were added at a concentration of either 10 x 10^5 or 5 x 10^5 to replicate wells of 1 x 10^4 HFF and to wells containing confluent HFM. Figures IV.7.3.1i) and ii) represent the percentage ^{3}H-C retention by HFM (y axis) and percentage specific ^{51}Cr release from HFF (x axis) by PBL from normal individuals and IMD patients respectively. The points on each graph therefore represent the % ^{3}H-Carnitine retention (HFM) and % specific ^{51}Cr release (HFF) by the same effector lymphocytes added per well. Table IV.7.3.1 (i and ii) gives the values obtained in these tests as well as the drug therapy which the IMD patients were receiving at time of study.

In order to test the correlation between myotoxicity, as measured by ^{3}H-Carnitine retention, and cytoxicity against HFF, as measured by specific ^{51}Cr release, straight line regression analysis (where $y = ax + b$) was applied to the results. From Fig. IV.7.3.1i), it can be seen that there was some correlation between myotoxicity and HFF killing ($p < 0.01$, $R = 0.73$) by effector lymphocyte populations from normal individuals. The value obtained for the slope of the regression line so derived ($a = -1.54$), indicated that fetal fibroblasts were less

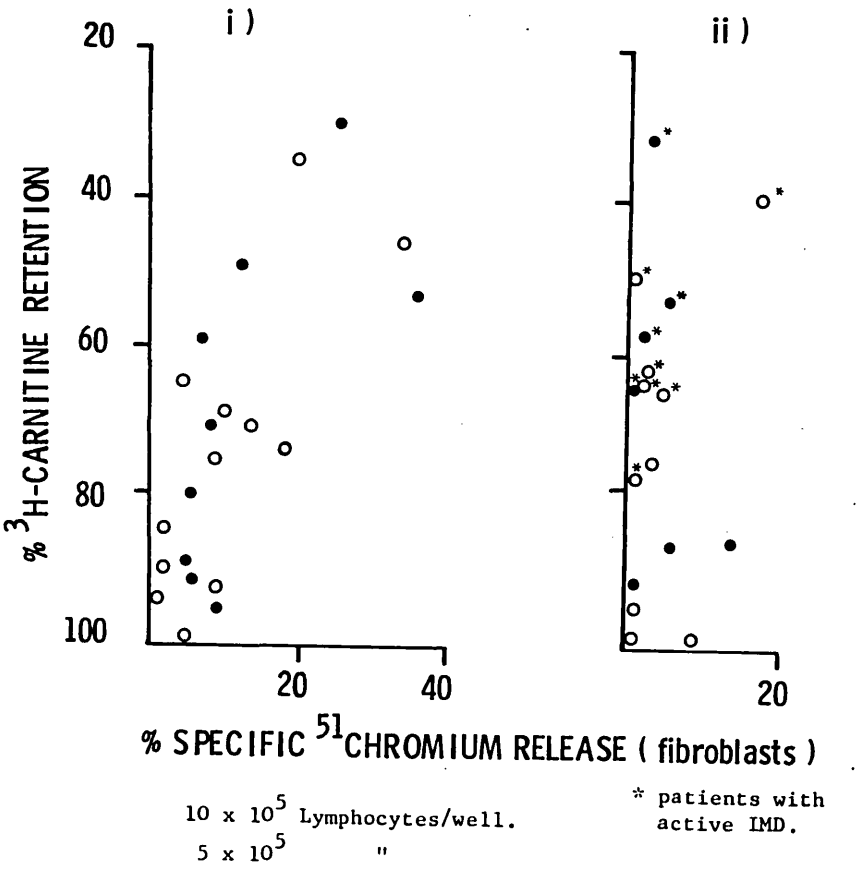


Fig IV.7.3.1 (i and ii)

The cytotoxic ability of lymphocyte populations from normal individuals (i) and IMD patients (ii), against HFF and HFM target cells. Results for individual patients are given in Tables IV.7.3.1 i and ii. Percentage ^{3}H-Carnitine retention by HFM is shown on the y- axis and percentage specific ^{51}Cr release from HFF on the x- axis. Solutions of equations derived following the application if straight line regression analysis to these results were as follows:

i) Normal subjects. $y = -1.54x + 88.40$; $R = 0.73$: $p < 0.01$

ii) IMD patients. $y = -0.47x + 73.35$; $R = 0.12$; $p > 0.06$

Initials	Age/Sex	Lymphocytes added per well (x 10^5) 10 HFF	10 HFM	5 HFF	5 HFM
KB	27M	36.3	53.4	32.2	45.7
MW	30M	4.4	79.1	2.4	84.9
"	"	6.5	58.8	5.7	63.5
KC	29M	8.6	95.6	5.6	100.0
PB	27M	11.8	48.7	9.4	68.9
"	"	*ND	ND	7.7	75.7
AN	25F	7.5	72.2	4.3	81.9
SW	24F	5.6	92.3	2.3	90.0
KE	29F	4.9	49.2	ND	ND
JC	28F	29.1	30.1	18.7	35.0
"	"	ND	ND	7.2	74.1
LK	47F	"	"	7.7	92.8
LW	33F	"	"	12.9	73.2
DG	35F	"	"	0.1	93.9

*ND: not done

Table IV.7.3.1.i

The results of percentage ^{3}H-Carnitine retention by HFM cultures and percentage specific ^{51}Cr release from HFF by lymphocytes from 11 normal subjects in 7 hour cytotoxicity experiments. Lymphocytes were added at a concentration of 10 and 5 x 10^5 per well in parallel experiments. Three of the inividuals were studied on 2 separate occaasions, approximately 6 weeks apart.

Initials of patient	Treatment Pred. mg/day	Other	Lymphocytes added per well ($\times 10^5$) 10 HFF	10 HFM	5 HFF	5 HFM
EK*	20		4.5	54.7	5.1	67.4
HM*	40		2.5	32.6	0.6	51.8
SH*	nil		1.7	58.1	1.8	65.0
PS*	"		ND	ND	16.7	41.0
SA*	"		ND	ND	2.5	64.1
VB*	60	Aza.	0.1	65.8	0.2	77.9
EK	30		5.7	86.8	2.9	77.0
PB	25		14.1	86.4	8.1	99.4
CS	15	Aza.	0.9	92.4	0.7	95.3
VB	60	Aza.	ND	ND	0.1	99.3

* patients with clinically active disease

ND: not done

Aza.: patients were receiving at least 100 mg Azathioprine/day

Pred: Prednisolone

Table IV.7.3.1.ii

Lymphocytes from 6 patients with clinically active IMD and 4 with inactive disease were used a effectors in cytotoxicity experiments with HFM and HFF as target cells. Patients EK and VB were tested when their disease was active and again when they were in remission. This table also gives the drug treatment that the patients were receiving when cytotoxicity measurements were made.

susceptible to lymphocyte-mediated lysis than were human fetal muscle cultures. In contrast, from Fig. IV.7.3.1ii) it can be seen that there was no correlation between cytotoxic responses against fibroblast and muscle cell targets by lymphocytes taken from IMD patients ($p > 0.6$: $R = 0.12$). When straight line regression analysis was applied to results of experiments performed using lymphocytes from the 6 patients with active disease at time of study, the formula $y = -1.26x + 62.3$ was obtained. No significant correlation between HFF cytotoxicity or myotoxicity was present ($p > 0.1$, $R = +0.47$). When results from patients whose disease was inactive at time of study, again, no correlation was found between myotoxicity and cytotoxicity against fetal fibroblasts ($y = -0.35x + 92.6$; $R = 0.22$; $p > 0.6$). However, it was observed that patients with active IMD and significant myotoxicity (< 80% ^{3}H-Carnitine retention) had values for HFF cytoxicity which were less than 5% specific ^{51}Cr release, with the exception of one measurement (Fig. IV.7.3.1ii). In Fig. IV.7.3.1i), values for HFF cytoxicity were all greater than 5% specific ^{51}Cr release when significant myotoxicity was shown by effector lymphocytes from normal individuals.

IV.7.3.2 Relative sensitivity of human fetal muscle and the K562 cell line to lymphocyte-mediated cytotoxicity

i) Experimental procedure and results: Figures IV.7.3.2i and ii represent the percentage ^{3}H-Carnitine retention by human fetal muscle (HFM) cultures (y-axis) and percentage specific ^{51}Cr release from pre-labelled K562 cells (x-axis) by peripheral blood lymphocytes from 10 normal individuals and 7 patients with IMD respectively. Five of the

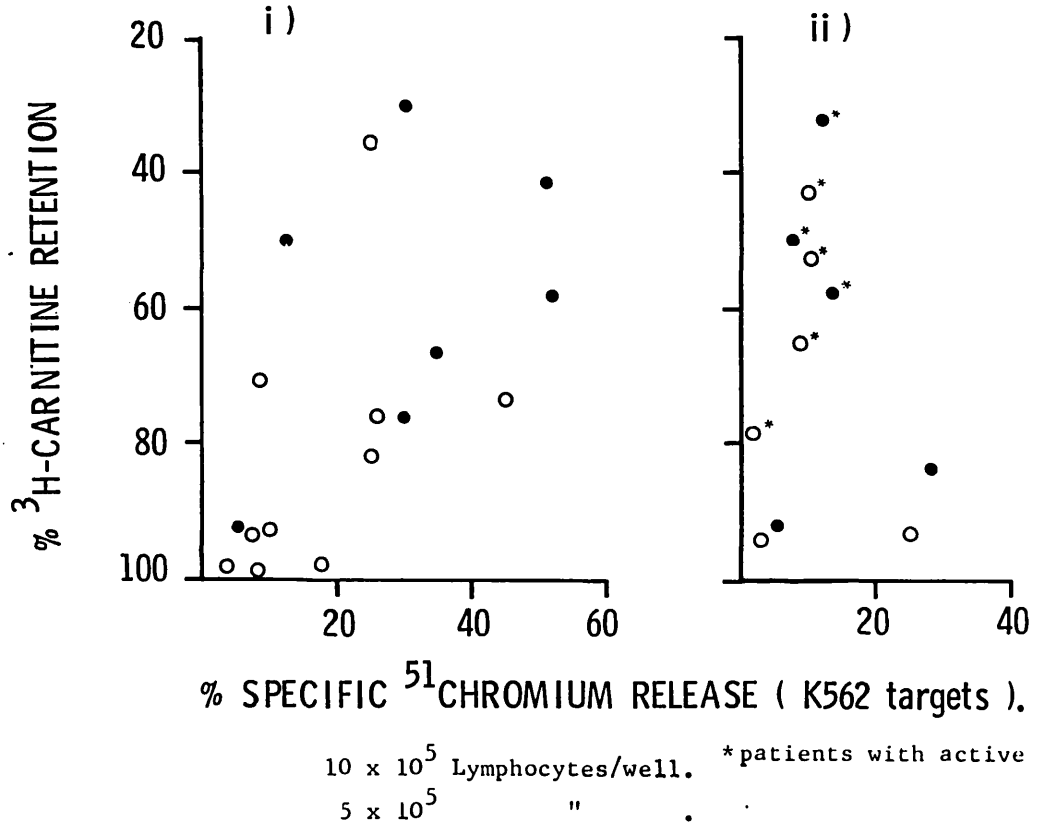


Fig IV.7.3.2 (i and ii)

The results shown here represent the cytotoxic ability of lymphocyte populations from normal individuals (i) and from IMD patients (ii), against HFM and K562 target cells in parallel experiments. Percentage ^{3}H-Carnitine retention by HFM is shown on the y- axis and percentage specific ^{51}Cr release from K562 cells on the x-axis. The results for individual subjects represented here are given in Tables IV.7.3.2 i and ii. The solutions of equations derived following the application of straight line regression analysis to these results were as follows:

i) Normal subjects: $y = -0.33x + 87.34$; $R = 0.46$; $p < 0.05$

ii) IMD patients: $y = 0.001x + 68.66$; $R = 0.01$; $p > 0.5$

		Lymphocytes added per well (x 10^5)			
		10		5	
Initials	Age/Sex	K562	HFM	K562	HFM
PD	26M	29.1	36.2	25.3	35.4
WT	25M	30.7	76.2	17.3	98.0
LW	31F	ND	ND	8.0	100.2
LK	45F	"	"	10.1	93.4
AR	28M	"	"	256.1	76.1
JC	28F	53.2	58.4	45.3	74.1
DG	35F	ND	ND	6.8	94.0
LC	22F	35.3	66.3	25.2	82.2
PL	42M	12.4	50.2	8.8	70.1
GW	32M	41.2	51.9	ND	ND

Table IV.7.3.2.i

The results of percentage ^{3}H-Carnitine retention (represented in 'HFM' column) and percent specific ^{51}Cr release from K562 cells (in 'K562' columns) by lymphocytes from 10 normal individuals, using a concentration of either 10 or 5 x 10^5 lymphocytes per well in parallel experiments.

Initials of patient	Treatment Pred. mg/day	Lymphocytes added per well ($\times 10^5$) 10 K562	10 HFM	5 K562	5 HFM
HM*	40	12.2	32.6	10.6	51.8
SH*	nil	14.2	58.1	8.5	65.0
PS*	nil	ND	ND	10.4	41.1
SA*	nil	"	"	33.0	64.1
VB*	60A°	"	"	1.5	77.9
HH	60A	28.4	84.3	25.2	93.9
CS	15A	4.6	92.4	2.2	95.3

* patients with clinically active IMD

°A patients were receiving at least 100 mg azathioprine per day in addition to prednisolone at indicated dosage

Table IV.7.3.2.ii

Lymphocytes from 5 patients with clinically active IMD and 2 with inactive disease were used as effectors against either HFM cultures or K562 cells. The results are represented similarly to those in Table 7.3.2.i, however the drug treatment which the patients were receiving at the time of testing are included.

patients with IMD had clinically active disease at the time of testing (depicted with an asterisk in Fig IV.7.3.2ii). Lymphocyte populations were added to wells containing either 1 x 10^4 ^{51}Cr labelled K562 cells or ^3H-Carnitine labelled HFM cultures at concentrations of either 10 x 10 or 5 x 10^5 cells per well. The points on each graph represent the percentage of specific ^{51}Cr release from K562 cells in 4-hour assays and the percentage of ^3H-Carnitine retention by HFM cultures in 7-hour assays at the same number of effector lymphocytes added per well for each estimation. In Fig. IV.7.3.2i, with the exception of two values (both obtained with the same effector lymphocyte population from one individual, P.L.), all significant (< 80% ^3H-Carnitine retention) myotoxicity results were associated with values for K562 cytotoxicity of greater than 20% specific ^{51}Cr release. Conversely, only two of the values obtained using effector lymphocytes from IMD patients (Fig. IV.7.3.2ii) gave a value greater than 20% specific ^{51}Cr release against K562 cells, although all patients with <u>active</u> disease had significant levels of myotoxicity. There was no correlation between myotoxicity and K562 cytotoxicity by lymphocyte populations from all the 7 IMD patients tested ($R = 0.01$, $p > 0.5$), nor when the results from the 5 patients with active disease were selected out from the group, ($y = -0.11x + 57.14$; $R = 0.07$; $p > 0.05$). However, there was significance at the 5.0% level between cytotoxic responses against K562 and human fetal muscle cultures, when effector lymphocyte populations were obtained from normal individuals ($R = 0.46$; $p < 0.05$).

IV.7.3.3 Lymphocyte-mediated cytotoxicity against human fetal fibroblasts and K562 cells: effect of removing $Fc_\gamma R$ bearing lymphocytes

i) Experimental procedure and results: The following experiments do not include studies on IMD patients because of the relatively large amount of blood required for each estimation. The cytotoxic ability of peripheral blood lymphocytes and of the lymphocyte population remaining following depletion of Fc_γ R bearing cells, against human fetal fibroblasts (HFF) and K562 cells, was measured in 12 normal individuals. The results, expressed as percentage specific ^{51}Cr release at an effector to target cell ratio of 50:1, are shown in Fig IV.7.3.3, where HFF's were used as targets in 7 hour assays, and K562 cells as targets in 4 hour experiments.

In Fig. IV.7.3.3, it can be seen that removal of Fc_γ R bearing lymphocytes from peripheral populations appreciably reduced cytotoxicity against K562 cells. Lymphocytes from 8/12 individuals gave values of greater than 10% specific ^{51}Cr release before depletion, compared with only 3/12 following removal of Fc_γ R bearing lymphocytes. Although human fetal fibroblasts are relatively insensitive to spontaneous cytotoxicity by peripheral blood lymphocytes, it can also be seen that removal of Fc_γ R bearing lymphocytes completely ablates detectable cytotoxicity against these cells.

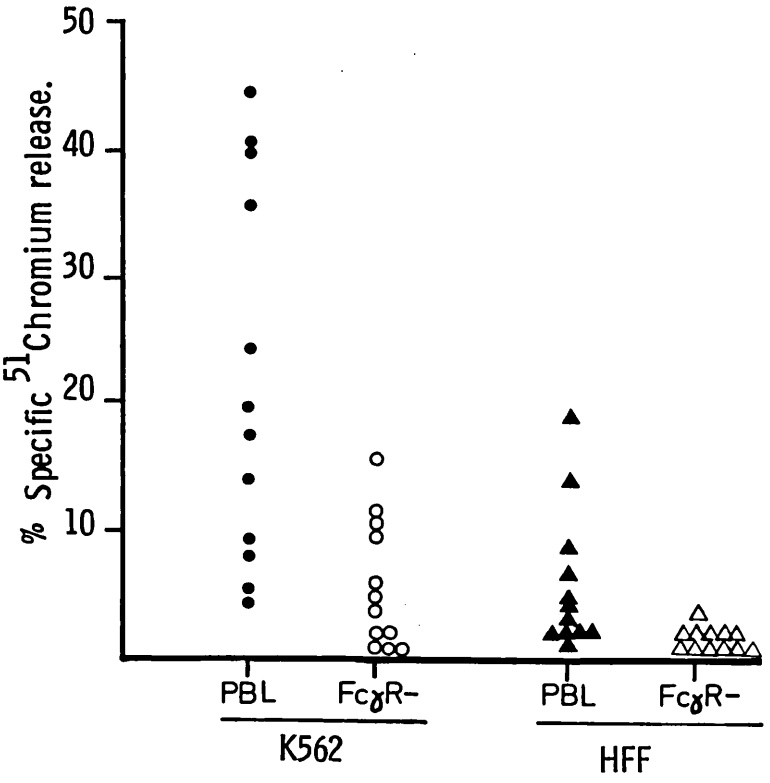


Fig IV.7.3.3

The effect of removing lymphocytes bearing Fc receptors for IgG on lymphocyte-mediated cytotoxicity. PBL or PBL depleted of lymphocytes with Fcγ -receptors, (FcγR-), from 12 normal individuals were tested for cytotoxicity against K562 cells in 4 hour experiments, or HFF targets in 7 hour experiments.

IV.7.3.4 Lymphocyte-mediated myotoxicity: effect of removing $Fc_\gamma R$ bearing lymphocytes

i) Experimental procedure and results: In the following experiments, human fetal muscle cultures derived from the same fetus were used as targets for both peripheral blood and Fc_γ R depleted populations from the same individual.

Fig. IV.7.3.4i represents the percentage ^{3}H-Carnitine retention by human fetal muscle cultures in 7 hour myotoxicity experiments following incubation with serial dilutions of peripheral blood lymphocytes from 12 normal individuals. Fig. IV.7.3.4ii depicts the percentage ^{3}H-Carnitine retention, following removal of $Fc_\gamma R$ bearing lymphocytes from these populations in parallel experiments. From Fig. IV.7.3.4i it can be seen that PBL from 5/12 individuals were significantly myotoxic (less than 80% ^{3}H-Carnitine retention) at 10 x 10^5 lymphocytes per well and 4/12 also at 5 x 10^5 per well. However, following removal of $Fc_\gamma R$ bearing lymphocytes (Fig. IV.7.3.4ii), no significant myotoxicity was apparent at any of the lymphocyte concentrations tested. Results are presented for each of these subjects in Table IV.7.3.4i.

Unfortunately, only 5 patients with IMD, 4 of whom were regarded as having clinically active disease, were able to be studied in this series of experiments. The myotoxic ability of peripheral blood and Fc_γ R depleted lymphocyte populations from these 5 patients were measured and the results presented in Figs. IV.7.3.4iii and iv respectively. Table IV.7.3.4ii gives values obtained for each individual in these experiments. Patients J.M., V.B. and M.H. were receiving 60 mg/day

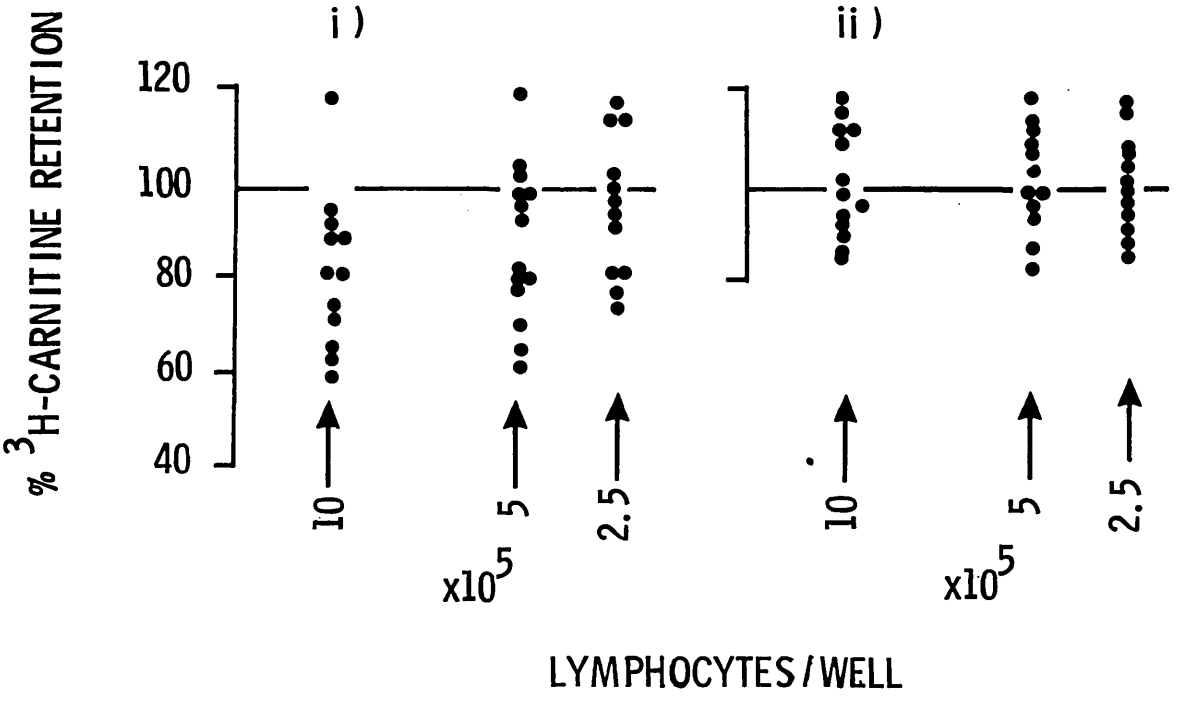


Fig IV.7.3.4 (i and ii)

These figures represent graphically, the results of lymphocyte-mediated myotoxicity by PBL and Fcγ -receptor bearing lymphocyte populations given in Table IV.7.3.4.i. Percentage ^{3}H-Carnitine retention by PBL from 12 normal subjects is shown in (i); and that measured following removal of FcγR-bearing lymphocytes in (ii).

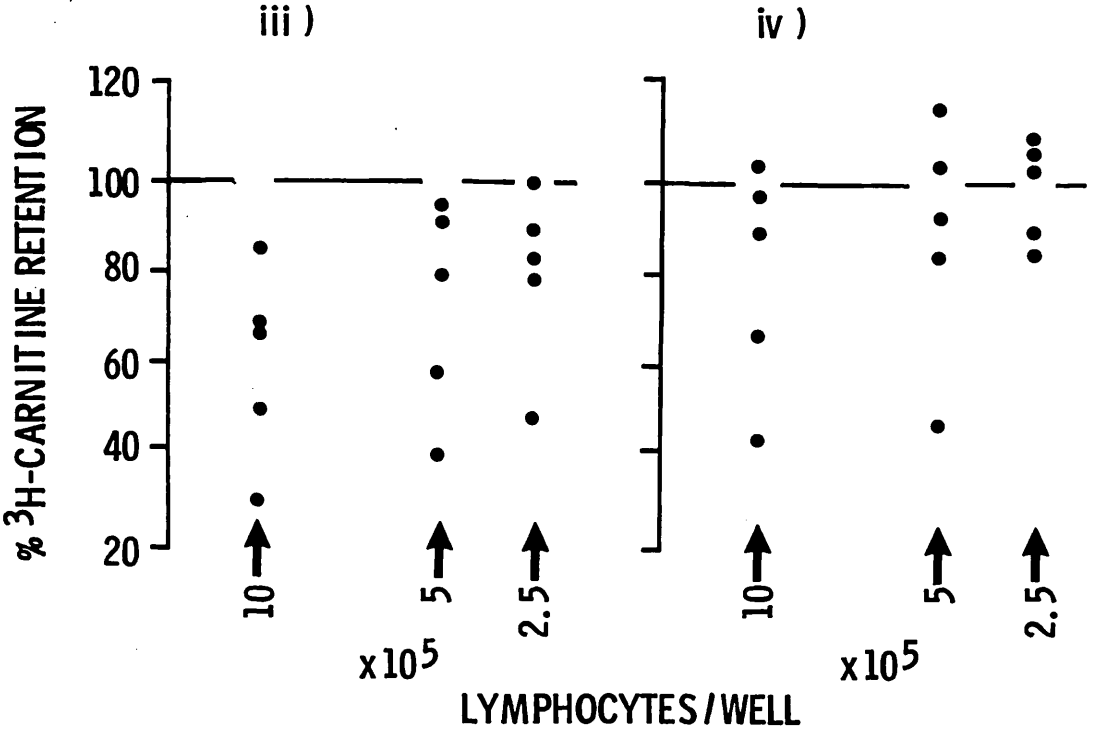


Fig IV.7.3.4 (iii and iv)

These figures represent graphically, the results of lymphocyte-mediated myotoxicity by PBL and Fcγ -receptor bearing lymphocyte populations given in Table IV.7.3.4.ii. Percentage ^{3}H-Carnitine retention by PBL from 5 IMD patients is shown in (iii); and that measured following removal of FcγR-bearing lymphocytes in (iv).

	% ^{3}H-CARNITINE RETENTION Number of lymphocytes added per well (x 10^{5})					
	10		5		2.5	
Initials	PBL	$Fc_{\gamma}R$-	PBL	$Fc_{\gamma}R$-	PBL	$Fc_{\gamma}R$-
WB	89	112	98	112	115	87
LW	66	76	65	73	80	92
JC	58	84	74	81	77	90
LB	72	83	79	83	75	84
JM	89	90	94	96	99	94
LC	80	110	102	119	101	107
PL	80	117	103	108	114	104
BW	66	110	82	111	81	111
AR	74	86	79	99	81	97
DC	93	94	99	99	97	100
CS	62	97	80	95	94	100

Table IV.7.3.4.i

PBL or lymphocyte populations depleted of $Fc_{\gamma}R$ bearing lymphocytes from 11 normal individuals were incubated with HFM in 7 hour myotoxicity tests. Percentage ^{3}H-Carnitine retention is shown for individuals, at three different lymphocyte concentrations as was determined for each effector lymphocyte population.

Initials of Patient	% ^{3}H-CARNITINE RETENTION Number of lymphocytes added per well (x 10^{5}) 10		5		2.5	
	PBL	$Fc_{\gamma}R$-	PBL	$Fc_{\gamma}R$-	PBL	$Fc_{\gamma}R$-
PS*	28	40	41	54	53	84
SA*	48	65	64	83	82	101
JM*	67	103	92	116	89	109
VB*	66	96	78	104	79	106
MH	84	89	94	92	99	87

* indicates patients with active inflammatory muscle disease.

Table IV.7.3.4.ii

PBL or lymphocyte populations depleted of $Fc_{\gamma}R$-bearing lymphocytes from 5 patients with clinically active and 1 with inactive IMD, were incubated with HFM in 7 hour myotoxicity tests. Percentage ^{3}H-Carnitine retention at three different lymphocyte concentrations was determined for each effector lymphocyte population.

prednisolone; V.B. and M.H. were also receiving 100 mg azathioprine per day. In Fig. IV.7.3.4iii it can be seen that PBL from 4/5 patients were significantly myotoxic at 10 x 10^5 lymphocytes per well and 3/5 at 5 x 10^5 per well. Following $Fc\gamma R$ depletion (Fig.IV.7.3.4iv), lymphocytes from 2 of the patients still showed significant myotoxicity at 10 x 10^5 lymphocytes added per well and one of these also at 5 x 10^5 per well.

These two patients also had the lowest values for percentage ^{3}H-C retention, 28% and 47%, by their PBL at 10 x 10^5 lymphocytes per well. It should be noted that the lowest values for percentage ^{3}H-Carnitine retention shown by PBL from normal individuals in this series of experiments at 10 x 10^5 lymphocytes per well were 58% and 62%, (Table IV.7.3.4ii). After removal of $Fc\gamma R$ bearing lymphocytes the percentage of ^{3}H-Carnitine retention by the depleted populations were 84% and 97% respectively at the same number of lymphocytes per well.

IV.7.5 Discussion

The cytotoxic responses of PBL taken from IMD patients and normal individuals against human fetal muscle and fibroblasts, and the K562 cell line were compared (IV.7.3.1 and IV.7.3.2). It was found that there were differences between lymphocytes from normal individuals and those from patients with clinically active IMD.

a) Cytotoxicity against HFM and HFF. There was a significant correlation between the cytotoxic ability of PBL populations from normal individuals when human fetal muscle and fibroblast cells were

used as targets in parallel experiments, although fibroblasts were less sensitive to lysis than muscle cultures in these 7 hour assays (IV.7.3.1). No such correlation was seen when effector populations were obtained from IMD patients, either in the active phase or in remission from their disease.

b) Cytotoxicity against HFM and K562. There was also a positive relationship between the cytotoxic responses of lymphocytes from normal individuals against the K562 cell line and cultured human fetal muscle. However, there was no significant association between the cytotoxic ability of lymphocytes from IMD patients against these two cell targets (IV.7.3.2). In addition, no association was found between cytotoxic responses against fetal muscle and K562 cells, when effector lymphocyte populations were derived from patients who had clinically active disease at the time of study (IV.7.3.2).

c) Although there was no statistically determined relationship between muscle cell killing and cytotoxicity against HFF or K562 cells by lymphocytes from patients with active disease, it was noted that significant myotoxicity was shown by their lymphocytes in association with relatively low values for cytotoxicity against HFF or K562 targets. This was in contrast with results of parallel experiments when lymphocytes from normal individuals were tested (IV.7.3.1 and IV.7.3.2). In normal individuals, an increase in cytotoxic activity against fetal muscle cells was accompanied by a concomitant increase in cytotoxicity against both HFF and K562 targets.

d) $Fc_\gamma R$ lymphocyte depletion. The depletion of $Fc_\gamma R$ bearing

lymphocytes from PBL populations obtained from normal individuals, almost totally removed the cytotoxic responses against HFF and HFM and significantly reduced those against K562 targets. Although few patients with IMD were studied, depletion of FcγR bearing lymphocytes removed some but not all *in vitro* myotoxicity from the effector lymphocyte populations.

In other studies, it has been shown that a proportion of the cytotoxicity against K562 cells by freshly isolated human PBL, is mediated by a lymphocyte population not expressing FcγR; the Fcγ-receptor, by definition, not being integral to NK-mediated cytotoxicity (Kay, Fagnani and Bonnard, 1979). Blockade of this receptor with Fc fragments does not appreciably inhibit NK activity. This is in contrast to antibody-dependent cellular cytoxicity (ADCC), which is mediated by a population of lymphocytes largely overlapping with NK cells, where ADCC is completely abrogated by this means (de Landazuri et al, 1979).

Cytotoxicity against HFF by human PBL is able to be more completely removed by depletion of FcγR-bearing lymphocytes (Saksela et al, 1979) as was found in these experiments (IV.7.3.3). Lymphocyte-mediated myotoxicity has not previously been studied, using an assay specific for *in vitro* muscle cell damage. However, from experiments reported here (IV.7.3.4), it was apparent that depletion of Fc γR-bearing lymphocytes was more efficient at removing myotoxic lymphocytes from PBL obtained from normal individuals than from those derived from patients with active IMD; the lowest values for ^{3}H-Carnitine retention by HFM for PBL populations from both groups, before Fc γ R-lymphocyte

depletion, being similar. However, the numbers of patients tested was too small to allow any further analysis regarding the phenotypic characteristics of myotoxic lymphocytes in IMD group.

e) Patients with inactive disease: The cytotoxic responses of 6 patients with clinically inactive IMD were included in these studies. Of these, 4/6 were receiving azathioprine (> 100 mg/day) in conjunction with corticosteroids. These patients all showed low levels of cytotoxicity against all three cell targets. Azathioprine is known to be a potent immunosuppresive agent (Lin, 1973). It acts on cells which are synthesising DNA and it is also directly cytotoxic to many cells, particularly those of the bone marrow. In vitro lymphocyte function is severely compromised in patients treated with Azathioprine (Bach and Dardenne, 1971) and NK activity is greatly reduced (Herberman et al, 1979).

V.B. was the only patient with clinically active disease who was receiving azathioprine when tested. Lymphocytes from this patient showed myotoxic activity in vitro. On consulting his case notes however, it was found that the two occasions when blood was taken for tests when his disease was "active", were both within a few days of his starting on azathioprine therapy. In conclusion, the effect of azathioprine therapy on in vitro lymphocyte-mediated cytotoxicity cannot be determined with any certainty from these adults. A discussion of the effects of corticosteroids on cytotoxic responses by lymphocytes from IMD patients is included in the Discussion of this thesis.

SECTION V

DISCUSSION

V DISCUSSION

CHAPTER I

V.1 NEW FINDINGS

V.1.1 Relationship to previous studies

The development of an *in vitro* assay for measuring specific muscle cell damage, presented two major problems which had to be overcome before clinical studies could be attempted.

Firstly, human fetal muscle had to be reliably established as healthy, monolayer cultures; preferably in a way in which replicate cultures would be available, and with little variation between these to allow accurate quantitation of immunologically mediated myotube damage.

When this work was initiated, a survey of the literature and consultation with those people having considerable experience in tissue culture techniques, regarding the culture of human fetal skeletal muscle, was not encouraging. It was claimed that myotubes would only grown and attain maturity if supplied with a collagen substrate. In addition, various 'special' culture conditions such as the use of low-zinc insulin, glucose and chicken embryo extract as medium supplements and a high (up to 50%) concentration of serum, (particularly horse serum or even cockerel or human placental serum) were also regarded as advantageous. The use of such culture conditions

would obviously compromise immunological studies. Culture vessels, such as Maximov chambers or roller tubes, had previously been used in studies of lymphocyte mediated myotoxicity. These require considerable quantities of tissue in order for the resulting monolayer to attain confluence and experiments would be limited with respect to the number of replicates available for study. As skeletal muscle tissue from 8-12 week human fetuses had been chosen by the author as the most suitable source of tissue for this work, the studies would be very restricted.

However, no great difficulty was encountered using standard tissue culture reagents and a suitable batch of fetal calf serum as culture medium for the establishment and maintenance of human fetal skeletal muscle, in 96-well flat-bottomed plastic microtitre plates. These cultures contained a high proportion of mature myotubes, obtained with the minimum of manipulation, and little variation between wells when derived from the same fetus. This allowed adequate replicate cultures to be used for the physiological and immunological studies undertaken; a considerable advance compared with cultures grown as explants or on glass coverslips.

V.1.2 ^{3}H-Carnitine as a specific label for cultured muscle cells

The second problem was to find a suitable radiolabelled molecule that might be taken up by myotubes in the mixed cell cultures derived from human fetal muscle, and likely to be rapidly released following myotube damage. It seemed that carnitine, available commercially in radiolabelled form, could be a suitable candidate. Although the kinetics of uptake and loss of ^{3}H-Carnitine by a transformed heart cell

line (Girardi heart cells) and by whole isolated rat skeletal muscle had been reported, there were not studies of these parameters available for cultured skeletal muscle. The physiology of H-Carnitine uptake and loss in human fetal muscle cultures, had thus to be established by the author. The kinetic studies, reported in Section III of this thesis, showed that carnitine is:-

a) selectively taken up by myotubes, as opposed to fibroblasts, in human fetal muscle-derived cultures.

b) lost from pre-labelled myotubes at a predictable rate over a period of time adequate for immunological assays to be performed.

c) able to be used as a specific muscle cell label in replicate cultures to a highly reproducible degree.

d) taken up by myotubes by means of a membrane-associated active transport mechanism. This was evident after applying Michaelis-Menton kinetics to experiments of carnitine uptake by HFM and the Lineweaver-Burke plots derived from these studies.

V.1.3 Application of ^{3}H-Carnitine uptake by cultured HFM to studies of lymphocyte-mediated myotoxicity

It was found that the development of the ^{3}H-Carnitine based assay for studies of lymphocyte mediated myotoxicity gave several advantages over existing methods and also allowed new observations to be made.

a) Dose response relationship between number of effector cells and percentage myotoxicity.

The test enabled the calculation of a dose-response relationship between the number of lymphocytes added per culture and muscle cell damage, calculated by the reduction in retention of ^{3}H-Carnitine by the cultures. In previous studies, this was not attempted; indeed it was difficult to ascertain the number of lymphocytes added in the assay. As an example, 'one drop containing between 1 and 6 x 10^{6} cells' were added to muscle cultures (Haas, 1980). The 'mononuclear' cell preparations in most early studies contained variable numbers of polymorphonuclear cells, platelets and monocytes (Currie et al, 1971; Haas and Arnason, 1974). In addition, in none of the previous studies were glass or plastic adherent cells removed from the effector populations. Such cells, predominantly monocyte/macrophage lineage cells, also have cytotoxic activity against cultured cells and thus the contribution of these cells to previous studies of 'lymphocyte' mediated myotoxicity is difficult to ascertain.

b) Number of lymphocytes required for test

A relatively small number of lymphocytes (8 x 10^{6} per test) were required for each estimate of myotoxicity, including four replicates at three lymphocyte concentrations per test, at least. This is particularly relevent when studying children and immuno-suppressed patients or those on long-term steroid therapy (adults and children), owing to the limitations on the quantity of blood available and the lymphopenia often present in these patients. It also allowed the

simultaneous measurement of the cytotoxic potential of the same lymphocyte population against targets other than muscle. This enabled the analysis of patterns of lysis by lymphocyte populations against muscle, fibroblast and K562 cells, resulting in the observation that lymphocytes from patients with active inflammatory muscle disease showed preferential lysis of myotube targets.

c) Target specificity

Both Dawkins and Mastaglia (1973) using a ^{51}Cr release assay, and Currie's group (1971), tested the cytotoxic potential of peripheral blood mononuclear cells from polymyositis patients against cell targets other than muscle. Dawkins and Mastaglia reported a slight, but not statistically significant degree of cytotoxicity against chick lung cells by patients with active disease. No appreciable cytotoxicity against either chick muscle or lung fibroblasts was demonstrated by effector cell populations from 41 control individuals. Currie et al., (1971) claimed that Type polymyositis patients (associated with connective tissue disease) had high levels of cytotoxicity, as measured by destruction of human or rat muscle and epithelial/fibroblast explants, against both types of target. Type ('uncomplicated') polymyositis patients' peripheral blood mononuclear cells seemed to destroy muscle preferentially as opposed to epithelial/fibroblast cultures. No normal individuals were tested. In conclusion, both groups of researchers had demonstrated some specificity for muscle, using their albeit crude assay systems. However, the results were not presented in a way which allowed analysis of the cytotoxic potential of lymphocyte populations from individual patients.

CHAPTER 2

V.2 INTERPRETATION OF LYMPHOCYTE-MEDIATED CYTOTOXICITY RESULTS

V.2.1 Introduction

This thesis re-assesses *in vitro* measures of muscle cell damage by lymphocytes from patients with inflammatory muscle disease. It was found that lymphocytes from these patients did show some specificity, in their cytotoxic response, for human fetal muscle using the ^{3}H-Carnitine based assay. There are several possible explainations as to the nature of this *in vitro* myo-specificity.

V.2.2 Possible identity of myotoxic lymphocytes

a) Myotoxicity as a result of increased NK cell activity in patients with inflammatory muscle disease.

Natural killer (NK) cells are a sub-population of lymphocytes which can be spontaneously cytotoxic, following recognition of as yet undefined determinants expressed on a wide range of allo- and xenogeneic cells. Particularly sensitive to NK-mediated killing are those which are transformed or infected with viruses, although normal and fetal cells can also be lysed. In these studies, it was found that peripheral blood lymphocytes from normal individuals could damage cultured fetal muscle and that the activity against muscle targets correlated, to some extent, with the killing of K562 cells. These latter targets being particularly sensitive to NK-mediated lysis and

commonly used as an index of NK activity by human lymphocytes. The myotoxic activity of lymphocytes from normal individuals was able to be ablated by removing cells bearing receptors for the Fc fragment of IgG, a marker present on approximately 85% of human peripheral blood lymphocytes with NK activity (Perussia et al., 1983). When lymphocytes from polymyositis patients with active disease were tested for in vitro cytotoxic activity, it was found that even when myotoxicity was significant, K562 killing was not necessarily increased. However, myotoxicity did seem to have some properties in common with cytotoxicity mediated by NK cells, being unrestricted with respect to products of the major histocompatability complex (Stern et al., 1980) and removable by depletion of the lymphocyte population usually containing the most activity. A property not usually attributable to NK cells, that of antigen-specificity, was however apparent. In humans, the target recognition structures on NK cells appear to be non-clonally distributed, but recent evidence (Pawelec et al., 1982 and Pawelec. G, personal communication, 1983) suggests that cytotoxic cells, which are not restricted by MHC products in their cytotoxic responses, can be isolated in vitro following culture of human PBL in interleukin 2 containing medium and with allogeneic stimulator cells. Cloned cells derived from such cultures could lyse certain cultured cell targets selectively and have no cytotoxic activity against K562 or other conventional NK targets. They were not recognising either major or minor histocompatability locus products on the target cells. It is thus possible that similar cells, with specificity for muscle cell antigens in this case, are generated in vivo in the course of IMD, particularly if the inflammatory response, against either muscle cell determinants or due to reactivation of viral infection of the muscle,

is increased during disease exacerbation. Although there is no direct evidence for the development of myo-specific NK-like cells in muscle from these patients, there are several interesting observations from animal model experiments which may support such an hypothesis. In a cardiotropic Coxsackie-B virus induced model of human myocarditis in mice, Wong, Woodruff and Woodruff (1977i and ii) and Huber et al., (1981), have demonstrated that splenic cytotoxic cells generated following infection of the myocardium, lysed uninfected cultured myocytes to a greater extent than other normal cultured syngeneic tissue cells or NK targets. In animals infected with a non-cardiotropic strain of virus or control animals, spleen cells lysed myocytes and NK targets to a similar extent. These experiments implied that cytotoxic cells with specificity for normal muscle cells could be generated following viral infection of the appropriate tissue in vivo. Further to these experiments, using a similar mouse model of Coxsackie B virus induced myocarditis, Gauntt et al (1979) demonstrated that the immunospecific cardiac antigens from infected mice were not virion-associated; their presence depended on infection of myocytes by this virus.

b) Myotoxicity mediated by alloreactive cells

There was also the possibility that the apparently myo-specific lymphocytes detected in the peripheral blood of IMD patients were recognising allogenic MHC products, seen as 'changed self' by these cytotoxic cells, on the cultured muscle cells. Precursors of cytotoxic lymphocytes which have specificity for alloantigens are present at high frequencies in normal PBL populations (Malissen et al, 1981). This

possibility was approached experimentally, by measuring the cytotoxic potential of effector lymphocyte populations simultaneously against skin fibroblast and muscle cells derived from the same fetus. As was the case when K562 cells, which do not express HLA antigens (Andersson, Nillson aand Gahnberg, 1979), were used as targets, lymphocytes from patients with active inflammatory muscle disease preferentially lysed the muscle cells. Lymphocytes from normal individuals also showed relatively low levels of activity against fetal skin fibroblasts, reflecting the insensitivity of these targets to NK lysis. However, when muscle killing was high, fibroblast killing was also increased to some extent. This was not seen on all occasions when lymphocytes from IMD patients were tested.

This does not disprove the possibility that MHC products are the target antigens, recognised by the apparently myospecific cytotoxic cells from the patients, as little is known concerning the expression of HLA antigens on fetal skin or muscle cells under these culture conditions.

Although not directly relevant, but thought to be worthy of further investigation, Beverley and his collegues at UCH have recently shown that the sarcolemma does not express MHC Class I antigens (determined by staining muscle biopsy sections with monoclonal antibody directed against the non-polymorphic chain of human Class I antigens). However, these antigens were expressed on the sarcolemma if the muscle fibre was damaged or inflammatory cells were adjacent to the fibre (Rowe, Isenberg and Beverley, 1983).

c) Myotoxic lymphocytes as examples of disease-specific cytotoxic cells

There are other examples of human disease where lymphocyte mediated cytotoxic specificity against allogeneic cultured target cells have been demonstrated. Examples include selective cytotoxicity against epithelial cells in inflammatory bowel disease (Kemler and Alpert, 1979) and rat islet cells in Type I (immune) diabetes (Charles et al., 1982) which do not require HLA syngeneity between effector and cell target. The identity of these cytotoxic cells, showing tissue-specificity related to the organ involved in the disease, have not been determined. As with the myotoxic lymphocytes detected in patients with inflammatory muscle disease, identification of the target structures being recognised is eagerly awaited.

d) Myotoxicity due to antibody-dependent cellular cytotoxicity

Although antibody-dependent cellular cytoxicity (ADCC), mediated by anti-muscle antibody and the lymphocyte sub-population bearing Fc_γ - receptors, was not excluded rigorously as a possible mediator of *in vitro* myotoxicity, such an explanation was not thought likely for several reasons.

For reasons related to the pathogenesis of the disease, there is no evidence for an increased incidence of anti-muscle cell membrane antibodies in patients with IMD, although a range of auto-antibodies to cellular cytoplasmic components have been detected. In muscle biopsies from IMD patients, the distribution of bound IgG and complement components is not necessarily related to the sarcolemma of

muscle fibres (Isenberg, 1983). Many plasma cells with cytoplasmic IgG and IgM were found between the muscle fibres in a careful study by Fulthorpe and Hudgson (1982). However, antibody was usually localised bound to intra cellular components of disrupted fibres and in the interstitial tissue. In Whitaker and Engel's (1972) much quoted study of muscle biopsies from children with IMD, antibody and complement deposition was usually limited to the walls of small blood vessels within the muscle tissue.

In experimentally-induced allergic myositis in animals, several investigators (Dawkins and Lamont, 1971; Fulthorpe and Hudgson, 1975) found that although antibody was associated with sarcolemmal membranes in muscle tissue and could bind to cultured skeletal muscle, it was not cytotoxic to muscle cells *in vitro* in the presence of complement. Dawkins, Eghtedari and Holborow (1971) reported a lack of correlation between anti-muscle antibody titre and myositis, suggesting that the two phenomena were different consequences of immunization and that antibody was not involved in the pathogenesis of the experimentally-induced disease.

In the human disease, Dawkins and Mastaglia (1973) showed that sera from patients with active IMD were not cytotoxic to cultured chick skeletal muscle in the presence of complement. In studies undertaken for this thesis, patient sera and complement were also not cytotoxic for cultured human fetal muscle (unpublished observations). These results could not be attributed to a lack of sensitivity of myotube membranes to complement-mediated lysis, as the studies of Friedlander and Fischman (1979) have shown that such cells are highly susceptible

to lysis by appropriate antisera and complement.

None of the patients included in this study had anti-sarcolemmal antibody in their sera, using standard immunofluorescence techniques. In addition, the way in which the assays were described performed made it unlikely that ADCC was operating. Effector populations were incubated at 4 °C overnight and then washed before use in 7 hour myotoxicity experiments. It is unlikely that sufficient anti-muscle antibody could be produced in such a period of time, given that there was no evidence of antibody against muscle cells by other means present in the patient.

It is my prejudice that the expression of receptors of $Fc_{\gamma}R$ on cells which can mediate both NK and ADCC activity, is more likely to reflect a means of regulation of the function of NK cells, rather than an *in vivo* effector mechanism via ADCC activity. Although NK mediated cytotoxicity is not inhibited in the presence of concentrations of up to 100 μg/ml heat aggregated IgG, modification of activity can occur if effector populations are pre-incubated with lower concentrations of aggregated IgG (Merrill et al., 1983).

V.2.3 Disease activity/effect of therapy:

Considerable confusion resulted from comparison of results of *in vitro* myotoxicity with clinical disease activity and the effect of therapy of these responses as described by other researchers. Haas and Arnason (1974) found that 9 out of 15 of their patients had peripheral blood mononuclear cells capable of releasing significant levels of creatine

phosphokinase from cultured rat muscle cultures, irrespective of activity or quiescence of disease, or of cortico-steroid therapy. Currie and co-workers (1971) did not present their data in a way in which information concerning the correlation between *in vitro* destruction of muscle cultures and disease activity in the patients could be obtained. By way of contrast, Dawkins and Mastaglia (1973) reported a positive relationship between activity of disease and ^{51}Cr release from cultured chick muscle. Cortico steroid therapy (10-70 mg per day) markedly diminished cytotoxicity.

In the experiments reported in this thesis, lymphocyte-mediated myotoxicity was found to correlate with disease activity in many of the patients studied. Measurement of *in vitro* myotoxicity also allowed quantitative comparison of disease activity in individual patients studied over a period of time. Unfortunately, little information regarding the effect of therapy on myotoxicity was gained, as few patients receiving immuno-suppressive therapy (azathioprine or other cytotoxic drugs) or high dose (> 80 mg prednisolone per day) steroids were studied (see IV.7.3.5) and were excluded as much as possible from investigations. This was because of the well described alterations of *in vitro* immune function resulting from these treatments (Schreiber, 1977). It can be seen from the children studied longitudinally in Chapter 4 of the Clinical Studies that, when azathioprine was introduced, on only one occasion did lymphocytes from one of these children show significant myotoxicity, and that test had been performed shortly after (2 days) Azathioprine treatment had begun, as was the case with VB, whose results were discussed in IV.7.3.5. The 5 patients studied in relapse or remission in Chapter 3 of that section were all

receiving approximately the same range of dosage of prednisolone when tested, in order to minimise drug related effects on *in vitro* lymphocyte function. The remainder of the adult patients studied, be they clinically active or inactive, were usually receiving less than 20 mg prednisolone per day, which does not dramatically reduce lymphocyte mediated cytotoxicity (Oshimi et al, 1980; Herberman et al., 1979).

V.2.4 Disease and normal controls

It was thought relevant to note that, although a number of normal individuals and patients with a variety of illnesses were tested for cytotoxicity against allo- and xenogeneic fetal tissues by other investigators, negative results were always found. This could simply be a reflection of the insensitivity of the assays used. As studies in this thesis have shown, peripheral blood lymphocytes from normal individuals can also kill human fetal muscle. Most of this cytotoxicity was probably mediated by Natural Killer cells, which would not be expected to be compromised in the patient control groups or normal individuals studied by these other workers. It would be expected that, given the wide range of NK activity present in the general population, at least a *few* of the controls would be high responders against such targets.

In retrospect, the patient control group chosen for these studies, which comprised mostly patients with SLE, was unfortunate, as it has been demonstrated that a proportion of these patients have a defect in Natural Killer cell activity, interferon production and interferon-induced augmentation of NK cytotoxicity (Fitzharris et al., 1983;

Neighbour, Grayzel and Miller, 1982). In most other rheumatological disorders studied, for example rheumatoid arthritis and scleroderma, unless systemic involvement is present in the patient, NK activity is within normal limits (reviewed by Cambridge, 1984).

CHAPTER 3

V.3 AETIOLOGY AND PATHOGENESIS OF INFLAMMATORY MUSCLE DISEASE

V.3.1 Introduction

In this chapter, the currently held views for the development of inflammatory muscle disease are discussed and the ways in which these may relate to the pathogenesis and immunological results found in patients. Suggestions for the means by which further investigation of these hypotheses may be conducted, are also given.

V.3.2 Persistent virus infection of muscle in IMD

Although infection of muscle with viruses or other microorganisms has not been demonstrated in many patients with IMD, it is possible that a proportion of cases are directly attributable to persistent infection of muscle tissue. Pathogenesis would presumably be the result of an immune response directed against infected myofibres which expressed either virus coded or virus induced cell surface antigens. Direct damage to the muscle by the infection would also occur.

It is difficult to demonstrate aetiological agents in affected tissue directly. A limited amount of tissue is available from patients for the isolation of potential organisms. Isolation of a wide range of likely myotropic viruses requires the ready availability of suitable susceptible cell lines. Experimental animals are required for the isolation of other potential pathogens, such as those of the Coxsackie

A group viruses. Consequently, most patients cannot be investigated in a way which rigorously excludes direct viral infection of muscle.

V.3.3 The detection of direct viral infection of muscle in IMD

The ideal tools for detecting viruses in muscle would be complementary DNA gene probes, specific for viral DNA or RNA sequences. The limited availability of such complementary DNA probes and tissue for study, inhibits the application of such an approach.

Even if virus could be isolated and viral genetic material detected in muscle, the argument that the virus was merely an opportunistic 'passenger' in already damaged muscle, could still apply.

V.3.4 Auto-antibodies to a potentially virus-induced cellular antigen

It has been claimed that the presence of an autoantibody, now usually referred to as Jo-1, in sera from many patients with IMD, is a specific marker for myositis. This statement stems from the observation that this particular autoantibody is not found in other connective tissue diseases, in which a wide range of antibodies against both nuclear and cytoplastic nucleic acids and proteins are found. Jo-1 antibody is reactive with the protein moiety of a histidine t-RNA synthetase complex (Matthews and Bernstein, 1983). This enzyme is known to be utilised by some viruses in virus-directed protein synthesis. Several explanations for the presence of such an autoantibody have been proposed by Matthews and Bernstein.

Firstly, viral infection of muscle may lead to increased utilisation of this complex. It was suggested that the association of immunogenic RNA with the protein antigen might contribute to a 'breaking of tolerance' to this particular intracellular component, thus allowing antibody production to continue following elimination of the virus.

Alternatively, the persistence of virus in muscle and the resultant unusually high concentration of histidine-t-RNA synthetase released from damaged cells could maintain an antibody response to this antigen. The production of the autoantibody under these conditions, would be analogous to the detection of anti-myosin and myoglobin antibodies in sera from patients with myositis and other myopathies. Until evidence of direct infection of muscle with viruses of the types which utilise this enzyme can be demonstrated in IMD, this hypothesis for the presence of Jo-1 antibody remains entirely speculative.

V.3.5 Autoantibodies and autoimmune disease

It is now thought that the function of immune system is characterised by constant referal to critical self-determinants. The implications of the finding that the production of antibody against any self-determinant is a normal feature of the immune system is that in autoimmune disease, other factors besides the ability to mount an immune response to such antigens are involved in the actual expression of disease. However, the production of autoantibodies in appreciable quantities and of an apparent specificity in particular diseases, for example anti-DNA antibodies in SLE and Jo-1 antibody in IMD, suggest that a defect in the immunoregulation of the production of certain

autoantibodies is related in some way to disease pathogenesis.

V.3.6 Autoantibodies in IMD: possible relationship to aetiology and pathogenesis

Studies of systemic lupus erythematosus have inferred that autoantibodies to self-determinants could be involved in the pathogenesis of autoimmune disease, either in immune complexes or as anti-idiotypic antibodies taking part in a defective immunoregulatory circuit. Some investigators have proposed that a similar sequence of events may be occuring in other connective tissue diseases, including IMD.

The presence of an antibody to an intracellular component such as histidine-t-RNA synthetase, in relation to possible aetiological and pathogenetic mechanisms in a relatively tissue specific disease such as IMD, however, is more difficult to explain.

It is possible that such an antibody may cross-react with a cell-surface component related to the distribution of the disease, in this case, a muscle membrane determinant. The continuing antigenic stimulus, coupled with inappropriate immunoregulation in individuals genetically pre-disposed to develop such responses, could result in antibody-mediated damage to the muscle cell membrane, with an inflammatory infiltrate localised to muscle. The increased amount of histidine-t-RNA synthetase released due to this insult could further propagate the chain of events involved. Immune complexes composed of the antigen, in combination with the Jo-1 antibody could potentiate

tissue damage in sites other than muscle.

A similar proposal for the development of IMD, has been suggested by Plotz (1983). He hypothesized that an antibody elicited in response to a virus during infection of muscle, may result in the production of an anti-idiotypic antibody, the combining site of which resembles the 'shape' of the initiating virus. This anti-idiotypic antibody could thus also recognise the muscle cell surface receptor for the virus. Continued antibody production to the viral agent could result in the synthesis of more anti-idiotypic/cell surface receptor antibody with concommitent muscle cell damage by the binding of the anti-idiotypic antibody to muscle and propagation of the disease.

The two hypotheses can be tested experimentally. However, their validation involves estimations of the cross-reactivity between anti-viral or anti-idiotypic antibodies and antibody specific for muscle cell surface determinants found in IMD sera. As there does not appear to be such an antibody against muscle cell membrane antigens, this might be difficult.

In addition, the pathogenesis of IMD, particulary in adult patients, does not seem to be that of an antibody or immune complex-mediated disease, despite the presence of immune complexes in sera from a large proportion of patients.

CHAPTER 4

V.4 INFLAMMATORY MUSCLE DISEASE: A PERSONAL VIEW

V.4.1 Model for the development of IMD

I have adopted the following general hypotheses for the development of IMD.

Viral infection of muscle, in an individual genetically predisposed to allow expression of IMD, may result in a lymphocyte-mediated response against muscle cells. The target antigens on muscle cell membranes could be either viral coded or induced determinants, present as a direct consequence of muscle cell infection, or, alternatively, following elimination of the aetiological agent, a normally expressed muscle cell specific antigen. In either case, the inflammatory response to muscle would by self-perpetuating. Identification of the antigen recognised by myotoxic lymphocytes from IMD patients could differentiate between the two proposed aetiologies.

V.4.2 Myotoxicity by lymphocytes recognising normal muscle cell antigens

The possibility that an immune response against muscle, through recognition of normally expressed muscle cell antigens, presupposes that such myotoxic lymphocytes are induced during the course of the initial infection. In this model, either:

a) Myotoxic lymphocytes are elicited in response to muscle cell antigens only in individuals who will subsequently develop IMD or

b) Myotoxic lymphocytes are able to be produced in all individuals following muscle infection, but suppression of these responses is ineffective in IMD patients.

These possibilities are difficult to test. However, the latter seems to me, to be the most likely.

The variability of the clonally distributed T-cell receptor for self-determinants can be considered to be analogous to that of antibody idiotypy. Lymphocytes from normal individuals have the ability to produce antibodies to self-determinants and there is no reason to doubt that precursor T-lymphocytes with antigen-binding receptors for muscle cell antigens exist in normal individuals. There is some evidence for such lymphocytes being induced by infection of muscle tissue, from studies of Coxsackie B virus myocarditis in mice. Cytotoxic lymphocytes with specificity for uninfected cultured myocytes do develop in the spleen of infected animals during the infection, which do not develop following infection of mice with a non-cardiotropic virus (Huber et al., 1981).

As the cytotoxic lymphocyte response has specificity for muscle cell determinants in this model and IMD is usually characterised by its restriction in organ distribution to muscle, it is unlikely that myotoxic lymphocytes would be merely the result of a polyclonal type of cytotoxic lymphocyte activation.

V.4.3 Immunoregulation of myotoxic lymphocytes

Antigen specific suppression of the anti-muscle cytotoxic lymphocyte response may be defective in patients who develop IMD, perhaps in an analogous fashion to the anti-idiotypic model proposed for the regulation of antibody production (Jerne, 1976).

An anti-idiotypic antibody or determinant present on the suppressor T-cell with specificity for the myotoxic lymphocyte receptor through which myotoxic responses are controlled, may be blocked by the presence of an antibody elicited during the initial virus infection. For example, such a 'suppressor' antibody could be Jo-1 auto-antibody. The site on the myotoxic lymphocyte whereby regulation of activity is mediated, and recognised by suppressor T-cells or antibody, would not be necessarily part of the muscle antigen recognition site.

There is no evidence for such an hypothesis in IMD. Until myotoxic lymphocytes are able to be isolated, and cloned T-cell lines established from these patients and the mode of their immunoregulation investigated, this hypothesis must remain purely speculative.

V.4.4 Genetic susceptibility

As it is proposed that defective immunoregulation allows the persistence of myotoxic lymphocytes in IMD, one might expect some linkage of the disease to genes controlling antigen recognition and control of the immune response. The genes coding for cell surface determinants involved in these functions are localised in the D region

of the HLA complex in man. There is a close association between the presence of Jo-1 antibody and possession of HLA-DR3 and HLA-DRW6. All IMD patients with this antibody in their sera were shown to have one or both of these D-allotypes in a study by Arnett and colleagues (1981), although a total of 6 patients only were investigated.

However, in the majority of patients, no linkage with D-allotypes has been found. This is perhaps not surprising when one considers the polymorphism of human immune response gene products. The genetic susceptibility to develop IMD may also be related to the expression of virus receptors and the nature of the immune response to infection elicited to particular organisms in different individuals.

V.4.5 Detection of myotoxic NK-like cells in IMD

In the model proposed for the development of IMD, I favour a breakdown in the control of cytotoxic T-lymphocyte responses to self-determinants on muscle in genetically predisposed individuals, as being the most likely pathogenetic mechanism for muscle cell damage.

Studies described in this thesis showed that lymphocytes, with specificity for muscle, were present in the peripheral blood of IMD patients. These lymphocytes were not restricted with respect to MHC antigens on HFM targets. Although they were thus NK-like in their activity, NK activity against cell targets such as K562 and HFF was not correspondingly increased. Myotoxicity correlated to some extent with the activity of inflammatory muscle disease in patients, as assessed independently by clinicians.

I suggest the following hypothesis for the presence of the preferentially myotoxic NK-like lymphocytes in patients with IMD. This is based on a consideration of possible differentiation pathways for NK cells and those which are thought to be followed by cytotoxic T-cells.

Induction of the T-cell cytotoxic response follows on their exposure to antigen presented by macrophages in the context of Class II determinants. Clonal expansion of selected precursor T-lymphocytes with target antigen specificity occurs and re-exposure to antigen at a later date results in rapid activation of such selected clones of lymphocytes and the characteristic anamnestic response. Competition amongst expanding clones of T-cells determines the avidity of antigen/MHC specificity (Wagner et al., 1981).

Although not dependent on thymic influence for differentiation and expression of activity, lymphocytes in the peripheral blood which have NK activity express some phenotypic determinants in common with T-cells. Most have low-affinity receptors for sheep red blood cells and approximately 50% express the the T_8 antigen. Such cells respond by proliferation to interleukin 2 (or T-cell growth factor), at a higher frequency than unstimulated T-cells, and develop other T-cell associated antigens such as T_3, as well as expressing class II determinants (Vose et al., 1982). The apparent maturity in differentiation of peripheral blood NK cells, shown by expression of the interleukin 2 receptor, (which has to be induced on T-cells by interleukin I or other factors), can also be inferred from the observation that NK cells, unlike T-cells, maintain a high degree of cytotoxicity in peripheral blood. Interaction with modified, virus-

infected or transformed cell surface antigens leads to a rapid increase in effector function. No pre-sensitisation or proliferation is necessary for this activation. A wide range of cell surface antigens are recognised by NK cells without the requirement of MHC antigens at either the induction or effector phase. However, compartmentalisation of NK cells, attracted to and trapped in sites of inflammation, may allow them to follow an independent differentiation pathway by proliferation; NK cells usually recognising target cells and developing reactivity without proliferation. In the environment of the muscle compartment, for example in a milieu of muscle cell specific antigens, it may be possible for antigen-driven differentiation and proliferation of NK cells to occur. This would perhaps explain the presence of mytotoxic lymphocytes with NK-like characteristics in IMD patients. This model presupposes that NK cells can recirculate through blood and extravascular compartments.

The possible contribution of such cells to muscle cell damage would be purely speculative. However, their presence in peripheral blood may provide an easily measured in vitro marker for inflammatory muscle disease, as has been suggested by the results reported in this thesis.

Although there is no evidence for such a sequence of events for the in vivo differentiation of NK cells, it is perhaps of interest that NK-like cytotoxic cells isolated from the synovial fluid of rheumatoid arthritis patients show different cytotoxic activity and patterns of lysis against target cells than peripheral blood NK cells from the same donor (Silver et al, 1982).

CHAPTER 5

V.5 FUTURE RESEARCH SUGGESTED BY STUDIES IN THIS THESIS

V.5.1 Direction of future studies in IMD

Studies of the origin, recirculation patterns and effector function of lymphocyte populations present in muscle tissue during IMD would be the most likely to give information about the pathogenesis of the disease. Isolation and identification of the target determinants on autologous muscle cells, recognised by myotoxic lymphocytes in individual patients (if such cells are indeed present) would perhaps shed some light on the aetiology of IMD.

The technical problems involved in such studies would limit these investigations. The following suggestions for further investigations in IMD would be possible to perform, using techniques currently available, and may, albeit indirectly, contribute to our understanding of possible pathogenetic mechanisms.

a) Cytotoxicity

Peripheral blood lymphocytes from IMD patients would be cultured in the presence of T-cell growth factors and membranes purified from muscle tissue to determine whether cytotoxicity against muscle cell targets exists at the clonal level.

Muscle biopsy specimens could also be cultured in the presence of IL2

and irradiated autologous PBL in an attempt to isolate activated lymphocytes infiltrating muscle tissue.

b) Proliferative response to muscle cell antigens

Although it is realised that specific antigen driven proliferation of lymphocytes *in vitro* can suggest only that such induction of T-cells is occuring *in vitro*, this line of research is perhaps worthy of following.

Using purified muscle membrane fractions, the frequency of muscle antigen responsive peripheral blood lymphocytes from patients could be determined, by limiting dilution analysis, in inactive and active disease. In the logical extension of such work, investigations of antigen-specific suppressor mechanisms by other lymphocyte populations, antibodies and serum factors could be studied using muscle-cell-antigen-specific proliferating clones derived from patients with IMD. The effect of drugs on suppressor mechanisms could also be analysed.

c) Muscle cell antigen expression

Although not directly related to the other lines of suggested research, it would be instructive to study the effects of virus infection and cytokines, such as interleukin 2 and interferon, on the expression of muscle cell surface antigens, in order to determine the way in which surface antigens are 'seen' by the immune system as a result of infection or inflammation.

V.5.2 Implications for therapy

Treatment of this and other autoimmune diseases is usually designed to dislocate the effector arm of the aberrant immune response. Because one is rarely able to specifically affect the immune response in this way, such treatment usually results in the appearance of many undesirable side effects. Thus, treatment must represent a balance between controlling disease activity and toxicity of therapeutic drugs.

By learning more about the pathogenesis and aetiology of IMD, one may be able to treat this condition by several approaches. For example:

a) the effector cells: if these are able to be distinguished from other lymphocytes selectively,more appropriate therapy could be designed

or

b) the target antigen(s): recognition and induction of the specific immune response to such an antigen or antigens could be selectively ablated

or

c) recirculation of lymphocytes: if the inflammatory infiltrate in muscle is mainly derived from lymphocytes entering muscle tissue and then being unable to recirculate, therapy aimed at changing such aberrant recirculation could be devised.

The first two approaches, may be able to be applied following clonal analysis of myospecific lymphocytes.

Initial high dose corticosteroid therapy is probably the most effective way of bringing about such a change in the recirculation pattern of lymphocytes, which is currently available.

The other most commonly used therapeutic agent in IMD, is azathioprine. This drug is usually considered to be most effective in B-cell disorders, although it does inhibit the growth of many somatic cells, including T-cells (Lin et al, 1973). In the light of my hypothesis for the development of IMD, an agent more specifically aimed at T-cells would seem to be more appropriate. Amongst those drugs in clinical use at the present time, Cyclosporin-A, which appears to have a more selective effect on effector T-cell induction, may be worth considering as an alternative to azathioprine.

V.5.3 Applications to research in other fields

The most obvious area in which the results of this work could be employed are those requiring a specific radiolabel to measure muscle cell damage or changes in metabolic activity in *in vitro* physiological studies.

As the kinetics of carnitine metabolism in cultured human skeletal muscle have been established, the effects of pharmacological mediators can be measured in relation to another parameter of muscle cell metabolism apart from measures of protein or RNA synthesis, for

example. Many researchers are already using the ^{3}H-Carnitine based myotoxicity assay in order to study a variety of immunological as well as physiological systems. Possibly the most clinically relevant is the establishment of a short-term (2 hour) assay to measure antibody-dependent cellular cytotoxicity against cultured rat muscle using lymphocytes, macrophages and sera from patients with myasthenia gravis. Preliminary results suggest that this is a more reliable measure of disease activity in these patients than estimation of antibodies against the acetylcholine receptor by the ELISA technique.

Carnitine deficiency diseases are another possibly fruitful area of application for these studies. The culture of muscle biopsies from patients and determining the nature of the carnitine-related defect using tritium-labelled carnitine as a 'marker', as well as the effect of various drugs on carnitine metabolism in vitro is an area in which the author is currently involved. These studies may have relevance for the treatment of some of the patients.

In conclusion, the problem of fibroblast 'contamination' in any experiments where cultured muscle is used has always precluded the use of such cultures in experiments requiring any degree of specificity for muscle. The ^{3}H-Carnitine based labelling technique for muscle, may contribute to the development of many more in vitro assays in a number of fields of research.

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Clin. exp. Immunol. (1981) **43**, 211–219.

The uptake of tritium-labelled carnitine by monolayer cultures of human fetal muscle and its potential as a label in cytotoxicity studies

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(Accepted for publication 17 October 1980)

SUMMARY

As a novel approach to the investigation of immune responses directed against muscle antigens in inflammatory muscle disease, the use of tritium-labelled carnitine as a selective marker for myotubes in monolayer cultures was investigated. Tritium-labelled carnitine was incubated either with monolayer cultures of human fetal muscle (which contain fibroblasts and myotubes) or with syngeneic monolayer cultures of human fetal fibroblasts. The rate of uptake and loss of tritium-labelled carnitine by muscle cultures was compared with that shown by fibroblast cultures; uptake being five times greater for muscle. Values for K_m and V_{max} were derived for both tissues in culture, the ratio K_m/V_{max} being 3·1 for muscle cultures and 0·46 for fibroblast cultures, indicating the presence of the active transport system for carnitine in the myotube membrane. Freeze-dried radioautographs of muscle monolayers, previously incubated with tritium-labelled carnitine, were made and confirmed the specific intra-tubular localization of the label. Fetal muscle monolayers, previously incubated with tritium-labelled carnitine, were used as targets in long-term cytotoxicity experiments into lymphocyte-mediated myotoxicity. Peripheral blood lymphocytes from patients with inflammatory muscle disease were shown to be myotoxic, but lymphocytes from normal individuals or those with non-inflammatory muscle disease were not. This system is likely to prove much more sensitive than those methods employing chromium-51-labelled cultures. Carnitine-based measures of myotoxicity closely followed the clinical activity of the disease in sequential studies carried out on one patient and the test shows considerable potential as a means of assessing myotube killing by lymphocytes on a per-cell basis.

INTRODUCTION

When skeletal muscle is disaggregated and grown in tissue culture, a monolayer of mixed cell type is formed. Initially, fibroblasts and myoblasts divide, the former adhering to the bottom of the culture vessel a little more rapidly than the latter. The myoblasts differentiate, fusing and forming myotubes, which lie upon and among a bed of fibroblasts. Several methods have been tried as a means of reducing the number of fibroblasts present in these cultures, such as pre-plating (Yaffe, 1968), or the exposure of the cells in the primary cell suspension to cytotoxic drugs (Moss, *et al.*,

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0099-9104/81/0400-0211$02.00.

1978) or X-irradiation (Friedlander, Beyler & Fischman, 1978). While these techniques are capable of reducing the numbers of fibroblasts considerably, elimination has not been achieved.

In certain inflammatory muscle diseases, such as polymyositis/dermatomyositis, serological and pathological evidence of immune responses directed against muscle have been described (Currie *et al.*, 1971; Pearson & Bohan, 1977). Lymphocytes from these patients have been shown to adhere to, and to cause disorganization of, cultured muscle monolayers (Currie, 1970). The identification of specific myotube damage by microscopy is handicapped by the difficulty of defining which cells in the culture have been affected by the addition of lymphocytes. The adherence, presumably antigen-specific, of lymphocytes from polymyositis patients to monolayer cultures of chick muscle, has been used as a measure of disease activity (Partridge & Smith, 1976). The numbers of adherent cells present in test lymphocyte populations were compared with those present in populations from patients with non-inflammatory muscle disease. The major disadvantage of this approach is that it gives no information about muscle cell damage. Alternatively, chromium-51 release from labelled muscle cultures, induced by the addition of lymphocytes from patients with inflammatory muscle disease, has been tried as a quantitative test of myotube-specific myocytotoxicity (Dawkins & Mastaglia, 1973). However, as the fibroblasts in the culture also take up chromium-51, the proportion taken up by myotubes is limited. Consequently, when myotube killing is greatest, there is only a small difference between non-specific and specific release with respect to myotube killing, but a much larger difference between specific release and maximum release. Chromium-51-based cytotoxicity studies are not, as a result, very sensitive measures of muscle-specific cytotoxicity.

An alternative approach would be to employ a compound selectively taken up by myotubes and likely to be released rapidly following myotube damage. Uptake by fibroblasts would need to be minimal. Carnitine (γ-amino-β-hydroxybutyric acid·3-methyl-betaine) is a promising candidate. Although it is found in a number of cell types, its concentration in muscle cells is particularly high (Greville & Tubbs, 1968). Furthermore, although cells other than muscle are capable of synthesizing carnitine, both cardiac and skeletal muscle cells depend upon carnitine produced by the liver for their supply, which they take up by means of a membrane-associated active transport system (Rebouche, 1977). The intracellular function of carnitine is to assist in the transport, across mitochondrial membranes, of long chain fatty acids, which are an important source of energy in muscle cell metabolism (Fritz & Marquis, 1965). Physiological studies of carnitine uptake and loss by whole and homogenized animal muscle preparations (Willner, Ginsburg & Dimaure, 1978) and by human myocardial cells from an established cell line (Bohmer, Eiklid & Johsen, 1977), have been published. Carnitine is available commercially in either tritium- or Carbon-14-labelled form.

We describe here experiments on the uptake of tritium-labelled carnitine by monolayer cultures of human fetal skeletal muscle, together with studies of its rate of metabolism and loss, the specificity of labelling in mixed myotube/fibroblast cultures and its potential as a marker for studies of lymphocyte-mediated myotube-specific cytotoxicity.

MATERIALS AND METHODS

Fetal tissue sources. Following approval by our ethical committee, human fetal muscle suitable for culture was made available to us, both from the Medical Research Council Human Tissue Bank at the Royal Marsden Hospital, London, and from the Institute of Obstetrics and Gynaecology at Hammersmith Hospital.

Tissue culture. Human fetal skeletal muscle was removed by dissection and placed in TC 199 medium (Flow), buffered with 0·35% sodium bicarbonate and containing 10% fetal bovine serum (Seralab) and penicillin and streptomycin (Flow). The tissue was finely chopped with a sterile scalpel and incubated at 37°C for 30 min in 0·025% trypsin in 0·5 mM EDTA (trypsin/versene). After digestion, the small muscle pieces were carefully teased apart, the larger clumps of tissue were allowed to sediment and the released cells incubated in 25-cm^2 plastic tissue culture flasks (Falcon). The non-adherent cells were removed and cultured in similar 25-cm^2 flasks. After 1 to 3 days of culture, the cells were detached by incubation with trypsin/versene, split and re-cultured in flasks. This treatment was repeated once or twice and the cells finally dispersed into flat-bottomed 96-well

tissue culture microtitre plates (Nunc) at a concentration of 2–4 × 10^5 cells/well. For radioautographic studies, 1 × 10^6 cells were finally dispersed into 35-mm-diameter plastic dishes (Falcon). Culture was continued for between 1 week and 10 days at 37°C and in 5% carbon dioxide in air, the medium being changed when necessary and the concentration of fetal bovine serum being reduced during the later stages of the culture period to allow myoblast fusion to take place, with the development of myotubes. The maturity of the cultures was judged by microscopy: only those cultures containing a confluent monolayer of healthy cells and an acceptable proportion of myotubes were used in the subsequent experiments. By similar methods, confluent cultures of skin fibroblasts were also obtained from each fetus and established in microtitre trays on the day preceding an experiment.

Labelling with carnitine. Tritium-labelled carnitine (^{3}H-C), specific activity 750 mCi/mmol (the Radiochemical Centre, Amersham), was diluted in TC 199 medium to a final molarity of 5·17 μmol/l. Twenty microlitres of this solution, containing 0·1 μCi of tritium, were added to 80 μl of TC 199 medium in each microculture well, which contained either a fibroblast or a muscle cell monolayer. For radioautographic experiments, 400 μl (containing 2 μCi) of ^{3}H-C solution was added to 600 μl of TC 199 medium in each 35-mm-diameter plastic dish and incubated for 24 hr. When making estimates of K_m and V, 0·1 μCi of ^{3}H-C per well, in different molarities of unlabelled carnitine, was added to parallel cultures of muscle cells, and incubated for varying periods of time.

Harvesting of carnitine-labelled cultures. After monolayers of fetal muscle or fibroblasts had been incubated with ^{3}H-C for an appropriate length of time, the cultures were washed three times in TC 199 medium, 50 μl of 4% sodium dodecyl sulphate were added to each well and the plate allowed to stand overnight at 4°C. The contents of each well were transferred to scintillation vials and each well washed with two 0·2-ml volumes of 0·85% saline, the washings being transferred also to the appropriate scintillation vials. To each vial was added 1·5 ml of Unisolve 100 scintillation fluid (Koch–Light), they were then mixed on a vortex mixer and allowed to stand overnight at 4°C. Emission of β-particles was measured photometrically on an LKB Rack-Beta scintillation counter.

Radioautography. Monolayer cultures of human fetal muscle in 35-mm-diameter plastic dishes, incubated with ^{3}H-C as described, were washed three times in TC 199 medium and snap-frozen by floating the dishes on liquid nitrogen. The frozen monolayers were dried in a freeze-drier (Chem-Lab) to a vapour pressure of 50 mTorr and kept under vacuum at 4°C in a glass desiccator. At radioautography, several 1-cm^2 pieces were cut from the bottom of each dish with a hot scalpel and placed in turn on a 1-cm-diameter stainless-steel post in a darkroom. A thin film of high-plasticizer K2 photographic emulsion (Ilford) was taken up on a 2-cm-diameter platinum wire loop mounted on a glass rod, and placed over each 1-cm^2 piece. The films were dried rapidly in air and exposed in light-proof boxes for 2 weeks. The preparations were developed in D19 developer (Kodak), stopped in 3% acetic acid and fixed in a 1:3 dilution of Amfix (Kodak), before staining the cell monolayers with haematoxylin and eosin.

Statistical comment. There was considerable variation in the number and size of myotubes between muscle cell cultures derived from different fetal tissue sources, although there was little variation between wells plated out from the same tissue source. As a result, it was necessary to normalize data obtained from different experiments on ^{3}H-C uptake, made using different preparations of fetal muscle. This might have been done by relating the total uptake of ^{3}H-C to the amount of DNA/well, but this method would not have allowed for differences in the proportion of myotubes to fibroblasts. It was decided to express the uptake of ^{3}H-C at any one time as a fraction of the uptake of ^{3}H-C at 5 hr in the same experiment. In this way, it was possible to pool information from different experiments.

Lymphocyte-mediated myotoxicity. Monolayer cultures of human fetal muscle were incubated with 0·1 μCi/well of ^{3}H-C as described above and the wells washed three times with culture medium. Purified peripheral blood lymphocyte populations, from patients with active or inactive muscle disease, non-inflammatory muscle disease or normal individuals, were prepared on Ficoll/Hypaque density gradients as previously described (Stern, 1979). Lymphocyte concentrations were adjusted to 10 × 10^6, 5 × 10^6, 2·5 × 10^6, 1 × 10^6 or 0·25 × 10^6/ml and 100 μl of each suspension added to each of five replicate muscle culture wells. These cultures were incubated for 18 hr at 37°C in 5% carbon dioxide in air, washed three times in medium and retained ^{3}H-C harvested as described above.

Lymphocyte-mediated myotoxicity was calculated according to the formula:

$$\left[\left(\frac{TR\ ^3H\ C - ER\ ^3H\ C}{TR\ ^3H\ C}\right) - \left(\frac{TR\ ^3H\ C - CR\ ^3H\ C}{TR\ ^3H\ C}\right)\right] \times 100,$$

where TR ^{3}H-C = retained ^{3}H-C in the absence of lymphocytes, ER ^{3}H-C = retained ^{3}H-C in the presence of test lymphocyte populations, and CR ^{3}H-C = retained ^{3}H-C in the presence of control lymphocyte populations.

RESULTS

Carnitine uptake and loss

In early experiments (not quoted), ^{3}H-C was added to monolayer cultures of human fetal muscle and its disappearance followed. Approximately half the label disappeared immediately from the supernatants and subsequent loss was minimal. However, most of the ^{3}H-C could be recovered from the monolayers by washing. It seemed possible that carnitine, which is hydrophobic, might non-specifically associate with membranes. Fig. 1 shows the results of experiments in which ^{3}H-C was incubated with muscle monolayers for 22 hr and replicate cultures harvested for scintillation counting after increasing numbers of washes. The recovery of ^{3}H-C fell markedly over the first two washes, but succeeding washes did not appreciably lower carnitine recovery from the monolayer.

In Fig. 2 the uptake with time of ^{3}H-C by fetal muscle and fetal fibroblast cultures seen in several different experiments has been pooled in the manner described in the Materials and Methods section. Approximately five times as much ^{3}H-C was recovered from muscle cell cultures as from fibroblast cultures at each interval of incubation. Fibroblasts took up ^{3}H-C steadily, but more slowly and the larger standard error for fibroblast uptake reflects the smaller amount of label recovered. The fractional values for the recovery of ^{3}H-C were subjected to regression analysis, the

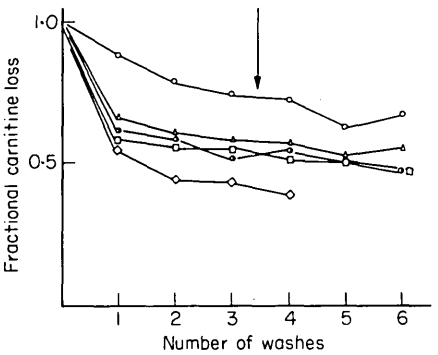

Fig. 1

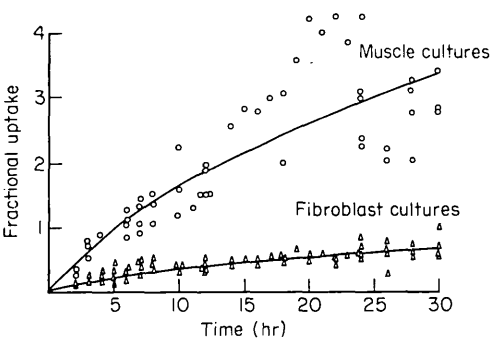

Fig. 2

Fig. 1. The influence of repeated washing with medium of human fetal muscle monolayers on the fractional retention of tritium-labelled carnitine. Each point represents the mean retention in five replicate cells. Five separate experiments are shown. Fractional retention is calculated as described in the text, assuming the retention *ab initio* to be unity. Regression analysis of each curve gave r values greater than 0·92 and standard errors of the mean less than 0·1, where $\log_{10} y = \log_{10} a + b\,x$, and a and b are constants. The arrow indicates the point at which cultures are normally selected for experimental use.

Fig. 2. The fractional retention of tritium-labelled carnitine by fetal muscle cultures (o——o) and by fetal fibroblast cultures (▵——▵) over 30 hr of incubation in eight experiments. The retention of ^{3}H-C in each experiment has been normalized to a value of 1 for muscle culture retention at 5 hr in the same experiment. The uptake of ^{3}H-C followed a power curve (the best fit for both types of monolayer by linear regression is shown) where, for muscle cultures, $y = 0{\cdot}7\ x^{0{\cdot}3}$ ($r = 0{\cdot}92$) and, for fibroblast cultures, $y = 0{\cdot}13\ x^{0{\cdot}49}$ ($r = 0{\cdot}84$). Standard errors of the mean were 0·25 and 0·27 respectively. Each value represents the mean of five replicate wells harvested after increasing intervals of incubation.

best fit obtained being that of a power curve, where $y = 0{\cdot}7\ x^{0{\cdot}3}$ with a correlation coefficient of 0·92 (r), for muscle cultures. Although retention increased progressively over the time period studied, an incubation period of between 8 and 12 hr resulted in sufficient ^{3}H-C retention to keep counting errors below 5%.

Observations on the rate of ^{3}H-C uptake and loss by muscle cell cultures from a single fetus were also made (data not shown). Replicate cultures were incubated with ^{3}H-C for 22 hr, washed once in medium and the culture then continued in medium containing 0·0588 nmol of unlabelled (cold) carnitine, equimolar with the ^{3}H-C solution. Before harvesting, cultures were washed three times in medium. In the presence of equivalent concentrations of cold carnitine, there were two phases apparent in the loss of ^{3}H-C from the monolayers: an early phase (over the first 8 hr), in which loss is slow, and a later phase, during which loss accelerates.

The effect of the presence of increasing molarities of cold carnitine upon the uptake of a constant dose of ^{3}H-C was examined (data not shown). When the muscle cultures were incubated for 2, 4, 5 or 6 hr, there was a linear relationship between $1/v$ (where v = pmol ^{3}H-C retained by cultures) and the ratio of cold to tritium-labelled carnitine, showing that the capacity for carnitine uptake by myotubes was not saturated. However, after 22 hr of incubation, $1/v$ seemed to reach a plateau at cold : tritium-labelled carnitine ratios of between 8 and 11. This suggests that, at this total carnitine dose (1 to 1·5 nmol), saturation may be achieved and, by extrapolation, that ^{3}H-C of less than 200 mCi nmol specific activity might not be sufficient to label the cultures under our conditions.

Fetal muscle cultures were incubated with 0·0588, 0·1176, 0·1764 or 0·2352 nmol ^{3}H-C for 5, 6 or 22 hr before harvesting and double-reciprocal (Lineweaver–Burke) plots made of ^{3}H-C recovery from these cultures and homologous fibroblast cultures. Regression analysis values, K_m and V_{max}, are shown in Table 1. K_m for muscle remained low at 0·1, while K_m for fibroblasts rose from 0·1 to 0·8. V_{max} for muscle was higher than fibroblast values, but not so much after 22 hr, when they were similar for both types of monolayer. However, K_m/V (1/slope) was consistently seven-fold higher for muscle cultures compared with fibroblast cultures. This shows a remarkable efficiency in carnitine uptake by muscle cultures, even though they contained a minority only of myotubes among a bed of fibroblasts homologous with the cells in the fibroblast cultures.

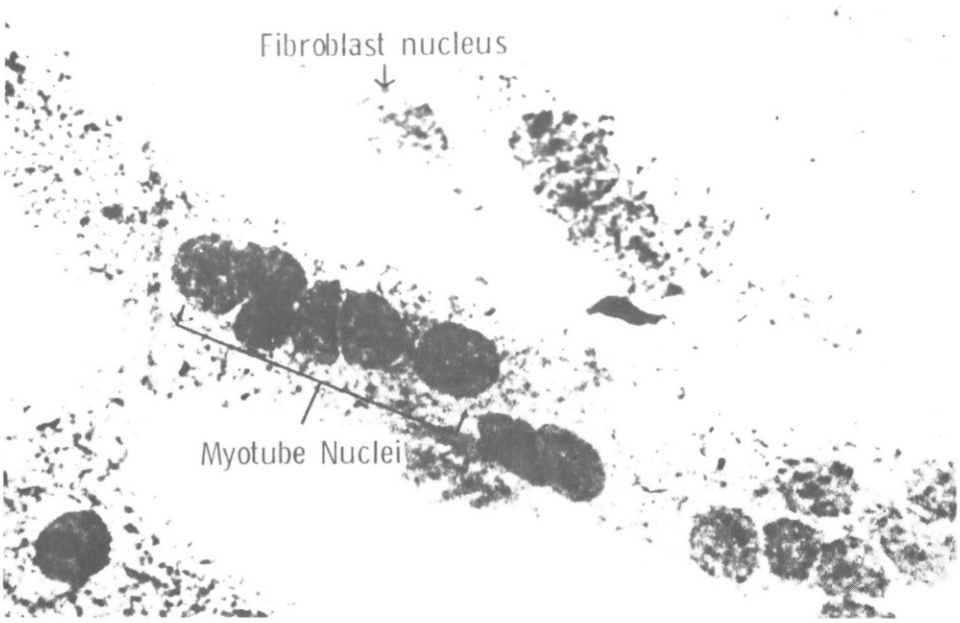

Fig. 3. Photomicrograph of a radioautograph of a freeze-dried fetal muscle monolayer after incubation with ^{3}H-C. The original print was made from a colour transparency. Silver grain deposition is closely related to myotube surfaces: the inter-myotubular spaces contain fibroblasts, as shown, and are almost free of precipitated silver.

Table 1. Linear regression parameters and values for K_m and V_{max} for double reciprocal plots of ^{3}H-C retention by fetal muscle and fetal fibroblast cultures, from the data shown in Fig. 5

	Hours of fibroblast culture			Hours of muscle culture		
Parameters	5 hr	6 hr	22 hr	5 hr	6 hr	22 hr
a_0	1·76	1·86	0·38	1·21	1·26	0·38
a_1	0·34	0·27	0·33	0·13	0·07	0·04
K_m/V $(1/a_1)$	2·94	3·70	3·03	7·69	14·29	25·00
s.e.m.	0·47	0·57	0·36	0·21	0·10	0·03
K_m	0·1932	0·1452	0·8684	0·1074	0·0555	0·1143
V_{max}	0·5682	0·5376	2·6316	0·8264	0·7937	2·8571
r	0·96	0·92	0·98	0·95	0·96	0·99
X-intercept $(1/K_m)$	5·18	6·89	1·15	9·31	18·02	8·75
Y-intercept $(1/V_{max})$	1·76	1·86	0·38	1·21	1·26	0·35

Values for linear regression are shown for the formula $y = a_0 + a_1x$, r = correlation coefficient and s.e.m. = standard error of the mean.

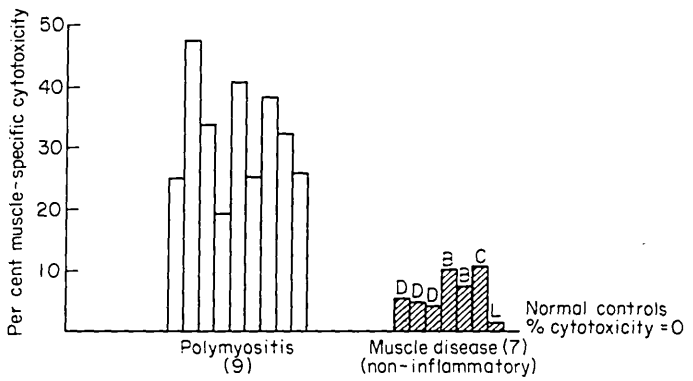

Fig. 4. Myotoxicity mediated by lymphocytes from 11 patients with clinically active polymyositis (□) and seven patients with non-inflammatory muscle disease (▨), compared with cells from normal individuals. Per cent myotoxicity was calculated as described in the Materials and Methods section: values for populations from normal individuals were therefore zero. The data given are restricted in each case to five replicate wells to which 5×10^5 lymphocytes were added. Mann–Whitney rank sum analysis, comparing active with non-inflammatory groups, gave $P \leq 0{\cdot}0025$. D = Duchenne muscular dystrophy, B = Becker dystrophy, C = congenital dystrophy (undiagnosed), L = limb girdle dystrophy.

Fig. 3 shows a photomicrograph made from a colour print of a radioautograph of a fetal muscle culture which had been incubated with ^{3}H-C. The inter-myotubular spaces, which contain fibroblasts, are almost free of silver deposition. Quantitation was not attempted, firstly because the multicellular nature of myotube origin made it difficult to relate myotube uptake to fibroblast uptake, and secondly because the designation of representative areas of the specimen for comparative counting might have introduced an element of bias.

Lymphocyte-mediated myotoxicity

Fetal muscle monolayers were incubated with ^{3}H-C and used as targets in studies of lymphocyte-mediated myotoxicity.

Fig. 4 shows the results of myotoxicity experiments, comparing patients with active polymyositis with those with non-inflammatory muscle disease. The difference in lymphocyte-mediated myotoxicity between these two groups was significant (Mann–Whitney rank sum, $P \leq 0{\cdot}0025$). Fig. 5 displays the change in lymphocyte-mediated myotoxicity sequentially in a single patient with

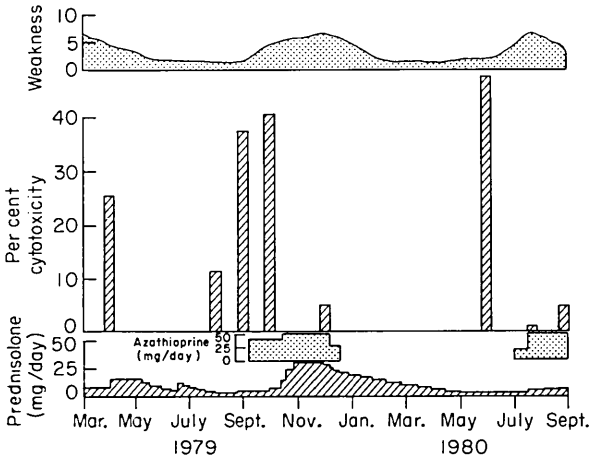

Fig. 5. Myotoxicity mediated by lymphocytes from a patient (C.S.) with dermatomyositis, tested on eight separate occasions and correlated with changes in muscle strength, according to a standard scale. Myotoxicity was calculated, at a dose of 5×10^5 cells well, according to the method described in the text, from five replicate wells.

dermatomyositis, correlated with her clinical state. Myotoxicity closely mirrored the clinical activity of her disease, as judged by her weakness.

DISCUSSION

These experiments were undertaken in a search for a suitable radiolabelled molecule that might be taken up by myotubes in mixed muscle cell cultures and released rapidly after immunological myotube damage. The washing experiment (Fig. 1) was carried out to see whether the release of ^{3}H-C from damaged myotubes could be detected in culture supernatants. The study showed that there was a tendency for extracellular ^{3}H-C to remain associated with the monolayer, probably in cell membranes. Because this ^{3}H-C can be removed effectively by three washes, in subsequent cytotoxicity experiments, including those reported here, ^{3}H-C retention by the monolayers was measured. All cultures were washed three times before solubilization with sodium dodecyl sulphate for scintillation counting, or before radioautography. We found that it is necessary to employ culture medium as the wash fluid, as the use of other isomolar solutions can lead to rapid detachment of myotubes during the washing process, and ^{3}H-C loss.

When ^{3}H-C was added to parallel cultures of either fetal muscle or fetal fibroblasts, muscle cultures retained the label to a greater extent (Fig. 2). Although myotubes represent only a minority of the cells in cultures of muscle, their presence was sufficient to create this substantial difference in ^{3}H-C retention. The true difference in the uptake of ^{3}H-C between myotubes and fibroblasts in the same monolayer culture will be greater than that shown in our experiments.

Because there was considerable variation in the size and proportion of myotubes to fibroblasts between cultures established from different fetal sources, direct comparison between the retention of ^{3}H-C in different experiments was not possible. A chemical estimate of the myotube mass present in each well might have been made, for example, by measuring the quantities of myosin or 3-methyl-histidine per well and expressing these values as ratios with respect to total DNA/well. However, as these microwell cultures were small, such techniques were not suitable owing to their insensitivity and the time required to carry them out. Normalization of ^{3}H-C retention to the 5-hr value, as described in the Materials and Methods section, was the chosen manipulation. Because there was little variation in the proportion of myotubes present in wells cultured from the same fetal source, the fractional retention of ^{3}H-C at different times reflected the activity of the membrane-associated active transport system for carnitine at different extra- and intracellular molarities of

carnitine. At the same time, these values were independent of the quantity of myotube plasma membrane present in different experiments. When results from different experiments were normalized in this way, ^{3}H-C uptake was shown to be similar for each culture. It was therefore possible to establish and label fetal muscle in a reproducible way with ^{3}H-C.

The values for K_m and K_m/V for muscle cultures compared with fibroblast cultures (Table 1) reflect the presence of an active transport system for carnitine in myotube membranes. In our experiments, ^{3}H-C uptake became more efficient as the period of incubation increased (K_m/V rose) but, as V_{max} for fibroblasts also increased at 22 hr, optimal differences in ^{3}H-C uptake between myotubes and fibroblasts at the molarity and specific activity used were achieved after 8 to 12 hr of incubation. Double-reciprocal regression analysis provides a convenient way of examining the efficiency of ^{3}H-C transport in these monolayer cultures. Such studies could prove helpful both in the standardization of ^{3}H-C-labelled cultures for cytotoxicity experiments in the investigation of hereditary muscle disease and in the other aspects of muscle physiology.

Radioautography was carried out on mixed muscle monolayer cultures to discriminate between the retention of ^{3}H-C by myotubes and the retention by fibroblasts. Because carnitine is a small, rapidly diffusing molecule, it was necessary to freeze-dry the labelled monolayers after washing. The morphology of the monolayers was well preserved, although crystals of culture medium, used as the wash fluid, remained on the surface and led to distortion of the photographic emulsion with faint, non-specific silver precipitation. However, tritium-induced silver grain deposition, by ^{3}H-C, was largely confined to the myotubes. This experiment provided technically independent confirmation of the preferential uptake of ^{3}H-C by the myotubes in these cultures.

The aim of these studies was to increase the sensitivity of measurements of lymphocyte myotoxicity in peripheral leucocyte populations from patients with inflammatory muscle disease. The representative cytotoxicity experiments shown (Figs 4 and 5) demonstrate that the method is sensitive enough for the purpose, and we have found it capable of showing lymphocyte myotoxicity on a per-lymphocyte basis. These studies have been made using 18-hr cytotoxicity experiments, but a standard 6-hr short-term killer cell (STKC) assay is under evaluation. Lymphocytes from some patients with dermatomyositis cause ^{3}H-C loss from muscle cultures in a fashion related to lymphocyte dose, while cells from others damage myotubes at all cell doses. Paradoxically, carnitine retention at high lymphocyte doses probably represents the redistribution of ^{3}H-C into lymphocytes adhering to the monolayer.

Although all myotoxicity experiments were carried out employing a range of lymphocyte doses, the small study in which lymphocytes from inflammatory and non-inflammatory muscle diseases were compared was analysed at a dose of 5×10^5 lymphocytes/well only. This dose gave the clearest significant differentiation between the two groups, but comparison of disease activity between patient samples and sequentially in the same patient will require myotoxicity to be measured on a per-lymphocyte basis, so that comparison by parallel-line assay may be performed (Michie, 1973). Adjustments for variations in the proportion of myotubes between experiments may be done by measuring the retention of ^{3}H-C at a given time for all cultures and normalizing the values as described here.

One patient (C.S.) was studied over an 18-month period (Fig. 4). She presented with typical clinical features of dermatomyositis and investigations revealed elevation of serum creatine phosphokinase and myopathic features on electromyography. Initial treatment with prednisolone led to remission, but her course began to show the fluctuations typical of this condition. Deterioration began after 5 months and initially continued, in spite of increasing doses of prednisolone and the later addition of azathioprine. There followed 6-month remission, when the dose of steroids was reduced. A subsequent myotoxicity test showed marked muscle killing and anticipated her relapse by 2 to 3 weeks. A recent increase in prednisolone dose and the addition of azathioprine has led to a further improvement. The clinical assessment of the activity of her disease was mainly subjective, but with respect to this new measure of myotube damage, it is encouraging to note the independent correlation between the severity of her muscle disease and the degree of myotoxicity shown by her lymphocytes.

No specificity controls were included in the myotoxicity studies reported here, such as the measurement of lymphocyte-mediated cytotoxicity against other cell types, because the principal

aim was to increase the sensitivity of the measurement of myotube damage in mixed cell cultures. The most interesting alternative cell for cytotoxicity studies is myocardial, and experiments on such cultures have begun. However, a more direct approach would be to define the molecule recognized by myotoxic lymphocytes on the myotube membrane. We have adapted the shearing technique developed by Crumpton and his colleagues (Crumpton & Snary, 1974; Crumpton *et al.*, 1978) to produce reasonably pure preparations of muscle cell membrane. We hope to purify muscle membrane components, such as glycoproteins, from these preparations and examine their antigenic role in inflammatory muscle disease.

This cytotoxic test, based upon the retention of ^{3}H-C by myotubes, has proved both reproducible and helpful in the assessment of disease activity in patients with polymyositis/dermatomyositis. The introduction of STKC tests promises to allow a more accurate definition in the study of disease groups and sequentially in the same patient. Finally, the identification of the target antigen recognized by myotoxic lymphocytes, by the use of blocking experiments, will be an important step in the understanding of the aetiology of inflammatory muscle disease.

The authors wish to express their gratitude to Dr G. R. V. Hughes, Professor A. P. Waterson and Professor V. Dubowitz for their encouragement and support and for permission to study patients under their care; to Professor J. H. Humphrey for his original and sagacious suggestions, and to Dr J. R. Sewell and Dr J. Z. Heckmatt for their clinical assistance. We would also like to thank Professor M. G. Elder and the Institute of Obstetrics and Gynaecology for their unstinting co-operation.

This work was supported by a grant from the Arthritis and Rheumatism Council of Great Britain and by a grant from the Muscular Dystrophy Group of Great Britain.

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