AN INVESTIGATION INTO THE PRESENCE OF COXSACKIE VIRUSES IN IDIOPATHIC INFLAMMATORY MUSCLE DISEASE

By David Andrew Hilton

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SUMMARY

Previous studies have claimed to have demonstrated the presence of coxsackie virus in skeletal muscle from cases of idiopathic inflammatory muscle disease (IIMD) by either culture, the finding of paracrystalline arrays at electron microscopy or hybridisation techniques to demonstrate coxsackie virus RNA in muscle. Serological studies have also linked coxsackie virus exposure to IIMD by the finding of elevated serum titres of antibodies to coxsackie viruses in cases of IIMD and murine models of myositis can be induced by inoculation of neonatal mice with coxsackie virus.

This study investigates the presence of coxsackie viruses in skeletal muscle from patients with IIMD using three techniques:

(i) *In situ* hybridisation to demonstrate the cellular localisation of virus RNA in muscle sections.
(ii) Reverse transcription-polymerase chain reaction (PCR) to search for small amounts of virus RNA in extracted total RNA from muscle.
(iii) Electron microscopy to search for the presence "virus-like" structures, in particular, paracrystalline arrays.

Oligonucleotide probes, each of 30 bases in length, were designed to be homologous to conserved regions of the coxsackie B virus genome. A "cocktail" of 6 probes was used in order to detect as many different virus strains as possible and to increase the sensitivity of detection. The probes were synthesised on an oligonucleotide sythesiser then labelled at the 3' hydroxyl group with digoxigenin-11-dUTP using terminal deoxynucleotidyl transferase. In addition control oligonucleotide probes to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA were designed and synthesised, in order to demonstrate RNA preservation within tissues.

To demonstrate the efficacy of these probes for the detection of virus by *in situ* hybridisation, mice, infected with several different strains of coxsackie virus, were obtained from the Coxsackie Reference Laboratory, West Park Hospital, Epsom. Formalin-fixed paraffin-embedded sections from these mice were hybridised with both coxsackie virus and control probes. Using the "cocktail" of coxsackie virus probes, all 11 strains of coxsackie virus were detected with no signal in the tissue from uninfected mice. Control probes demonstrated RNA preservation in all mice. The distribution of virus RNA largely matched that of the morphological changes.

In the coxsackie A virus strains (A5, A7, A8, A10, A16 and A21), virus was distributed widely thorough out skeletal and cardiac muscle, with a focal distribution in brown fat. The coxsackie B virus strains (B1-5) had a more limited distribution in skeletal muscle, predominantly involving the front limb girdle, head and neck muscles. In addition, with all coxsackie B virus strains, there was focal infection of the myocardium, salivary glands, pancreas, thyroid, liver, brown fat, hair roots and central nervous system.

26 cases of IIMD were selected from the files of Leicester Royal Infirmary and Kettering General Hospital. The case notes and histology of all were reviewed to ensure that they fulfilled the published criteria for diagnosis of IIMD. Sections from the cases (either paraffin embedded or cryostat) were used for *in situ* hybridisation with coxsackie and control probes. All cases showed evidence of RNA preservation using control probes to mitochondrial rRNA (which was found to be more useful as control probes than the G3PDH probes). With the coxsackie probes, 9 cases showed signal within interstitial mononuclear cells and 5 cases showed focal signal within muscle fibres. In both of these instances the signal did not disappear with RNase predigestion of sections and identical signal was seen when using a range of other oligonucleotide probes which had been designed to detect unrelated RNA species. The signal within interstitial mononuclear cells appeared to be within mast cells and disappeared following acetylation of tissue sections, suggesting that a charge related phenomenon was causing the oligonucleotide probes (negatively charged) to bind to basic substances within mast cell granules. This mast cell signal had a very similar appearance to the signal previously described by others in cases of IIMD, both with probes to coxsackie virus and Theiler's murine encephalomyelitis virus, when it was thought to be genuine hybridisation. The muscle fibre signal could not be removed or blocked by any of the methods tried and was only seen in cases of inclusion body myositis (IBM). The distribution of signal (around vacuoles and within nuclei) suggests a relationship to the filamentous inclusions of IBM, which occur at the same location, although the precise nature of this binding was not determined. None of the 26 cases of IIMD examined showed any evidence of coxsackie virus RNA using *in situ* hybridisation.

In order to confirm that the coxsackie oligonucleotides were able to detect strains of coxsackie virus infecting human tissue, post-mortem paraffin-embedded material from 10 cases of childhood myocarditis was examined by *in situ* hybridisation. 2 of the cases had evidence of virus present within myocardial fibres, predominantly in

areas of inflammation and necrosis. In one of these positive cases coxsackie B2 virus was cultured from the stool of the patient. These *in situ* hybridisation findings confirm a direct pathogenic link between coxsackie virus and some cases of childhood myocarditis.

PCR primers were designed to detect coxsackie virus RNA and G3PDH mRNA, following reverse transcription. A method for extraction of RNA from paraffin-embedded tissue was developed and using this method the coxsackie primers were shown to detect all 11 of the coxsackie virus strains following reverse transcription-PCR, from tissue extracts of sections of infected mice. Virus RNA was detected using as little as 0.01% of the extract from a single 10μm paraffin section. Virus RNA was also detected in the 2 cases of human myocarditis which were positive by *in situ* hybridisation. Of the 26 cases of IIMD examined, none showed evidence of coxsackie virus RNA within the muscle by reverse transcription-PCR, even when using internal "nested" primers to further increase sensitivity. These findings are in agreement with 2 recent PCR studies which also failed to demonstrate virus in any of over 80 cases of IIMD. The control primers to G3PDH mRNA produced strong bands in all 11 frozen blocks of tissue examined, but more variable results in formalin-fixed cases, with positive bands present in only 9/19 cases.

Electron-microscopy of 17 cases did not reveal any of the paracrystalline arrays that have been previously described in some cases of IIMD.

This study has demonstrated the ability of digoxigenin labelled oligonucleotide probes to detect a wide range of coxsackie virus strains in formalin-fixed murine and human post-mortem tissues by *in situ* hybridisation. The absence of detectable coxsackie virus RNA, using both *in situ* hybridisation and reverse transcription-PCR, in any of the cases of IIMD examined makes it unlikely that persistent infection of skeletal muscle by coxsackie virus is important in the pathogenesis of this disease. However, the possibility of IIMD being an autoimmune process, "triggered" by coxsackie virus infection, remains.

DEDICATION

I would like to dedicate this work to Angela.

ACKNOWLEDGEMENTS

Dr A Flowers for arranging for the coxsackie infected mice to be produced at the Coxsackie Reference Laboratory, West Park Hospital, Epsom and for Dr S Chambers and Carol Day for kindly agreeing to do this free of charge.

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The Arthritis and Rheumatism Council and Trent Regional Health Authority for funding of my salary and all the equipment/reagents used for this study.

DECLARATION

The majority of the practical work carried out in this study was performed by myself mainly during the period of registration, including the following: design of oligonucleotides for use as probes for *in situ* hybridisation and PCR primers; cutting of paraffin and cryostat sections; developing methods for RNA extraction from both paraffin and cryostat sections; labelling of oligonucleotide probes; performing all the *in situ* hybridisation and PCR experiments, including Southern blotting; and all the word processing of the text.

Work not performed by me is detailed below:

(i) The preparation of sections for electron-microscopy was carried out by the staff in Electron Microscopy at Leicester and Bristol.
(ii) The production of mice infected by different strains of coxsackie virus was carried out by Carol Day at the Coxsackie Reference Laboratory, Epsom.

Signed

David Andew Hilton May 1994.

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LIST OF ABBREVIATIONS

A	adenine
AMV	avian myeloblastosis virus
ANA	anti nuclear antibody
ANF	anti nuclear factor
BCIP	5-bromo-4-chloro-3-indolyl phosphate
C	cytidine
cDNA	complimentary deoxyribose nucleic acid
CPK	creatine phosphokinase
CXB	coxsackie B group virus
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
DM	dermatomyositis
DMF	dimethylformamide
DNA	deoxyribose nucleic acid
dNTP	deoxynucleoside 5'-triphosphate
ds DNA	double stranded deoxyribose nucleic acid
DTT	dithiothreitol
dUTP	deoxyuridine 5'-triphosphate
EDTA	ethylenediamine tetra-acetic acid
ENA	extractable nuclear antigen
FITC	flourescein isothiocyanate
G	guanine
g	gram
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
H&E	haematoxylin and eosin
IBM	inclusion body myositis
IIMD	idiopathic inflammatory muscle disease
IMS	industrial methylated spirits
JDM	juvenile dermatomyositis
Kb	kilobase
KGH	Kettering General Hospital
l	litre
LRI	Leicester Royal Infirmary
m	meter
M	molar

MND	motor neuron disease
MoMuLV	moloney murine leukaemia virus
NBT	nitroblue tetrazolium
n	nano
OD	optical density
p	pico
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
PM	polymyositis
PE	phosphate ethylenediamine tetra-acetic acid
RhF	rheumatoid factor
RI	replicative intermediate
RLP	ribosomal landing pad
RNA	ribose nucleic acid
RNase	ribonuclease
RT	reverse transcription
SDS	sodium dodecyl sulphate
SSC	standard citrate saline
SSDNA	salmon sperm deoxyribose nucleic acid
ssDNA	single stranded deoxyribose nucleic acid
T	thymidine
TAE	Tris acetate ethylenediamine tetra-acetic acid
TBE	Tris borate ethylenediamine tetra-acetic acid
TBS	Tris buffered saline
TdT	terminal deoxynucleotidyl transferase
TE	Tris ethylenediamine tetra-acetic acid
Tm	melt temperature
TMEV	Theiler's murine encephalomyelitis virus
TPE	Tris phosphate ethylenediamine tetra-acetic acid
μ	micro
U	uracil
UP	ultrapure
UTR	untranslated region
UV	ultra violet
V	volts

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PART 1

INTRODUCTION

1. IDIOPATHIC INFLAMMATORY MUSCLE DISEASE

1.1 Introduction

Polymyositis was first described by Wagner in 1863 (Wagner, 1863). The additional involvement of skin was described later (Unverricht, 1887). This spectrum of muscle disease is known as idiopathic inflammatory muscle disease (IIMD) and is one sub-group of inflammatory myopathies (myositides) which are all characterised by the presence of inflammation within skeletal muscle (see Table 1).

Table 1 - Inflammatory myopathies.

Idiopathic

1. IIMD - including polymyositis, dermatomyositis, inclusion body myositis, myositis associated with connective tissue disorders or malignancy.

2. Localised nodular myositis.

3. Eosinophilic myositis.

4. Myasthenia gravis.

5. Focal myositis.

6. Granulomatous myositis (may be associated with sarcoidosis or thymoma).

Infective

1. Viral myositis - coxsackie, influenza, Bornholm disease, benign myalgic encephalomyelitis, Echo virus dermatomyositis in agammaglobulinaemia, human immunodeficiency virus.

2. Bacterial - Tropical pyomyositis.

3. Parasitic - toxoplasmosis, cysticercosis, trichinosis.

As well as those listed above other muscle diseases such as fascio-scapulo-humeral dystrophy, may have a prominent inflammatory component.

For the purposes of this thesis those conditions listed as IIMD will be grouped because of similarities in clinical presentation and pathological features. These would have previously have been classified as the polymyositis-dermatomyositis complex.

IIMD may be defined as a primary inflammatory disease of skeletal muscle of unknown aetiology, characterised by symmetrical weakness of the limb girdle muscles. There are varying degrees of skin involvement (dermatomyositis) in approximately one third, and various other clinical features may be seen (see Table 2).

The overall incidence is 5/1000000, with a female predominance of about 2:1, except in cancer associated myositis (sex ratio 1:1) and inclusion body myositis (male:female = 3:1). There is a bimodal age distribution with age peaks of 5-14 and 45-64. A seasonal distribution has been noted with childhood cases, with a cluster in February-April (Medsger *et al*, 1970). Some cases may also present in the post-partum period (Steiner *et al*, 1992).

1.2 Clinical features

Disease progression is usually in weeks or months, rather than years, although it can sometimes be quite rapid (acute myositis). In such acute cases there may be rapid, generalised weakness with constitutional upset, and in "hyperacute" myositis oedema of muscles may occur.

Weakness often affects pelvic and shoulder girdle muscles producing a proximal myopathy, with symptoms such as difficulty in climbing stairs or lifting the arms above shoulder level. Symptoms are usually worse in the legs and muscle atrophy is rare early in the disease. The neck flexors may be involved causing difficulty in lifting the head when lying down. Facial muscles are not usually involved, which is helpful in distinguishing IIMD from other conditions such as muscular dystrophy. Ocular muscles are almost never affected. Involvement of the posterior pharyngeal muscles can produce a picture of partial bulbar palsy with dysphagia and dysphonia. The respiratory muscles are only rarely affected.

Contractures may occur in advanced disease, particularly in the biceps, but only rarely early on. Calcinosis of muscles can occur, most commonly in childhood dermatomyositis.

IIMD may present as multiple painful nodules over the limbs, which if left untreated may progress to diffuse disease (Cumming *et al*, 1977). This presentation should be differentiated from focal myositis (Heffner *et al*, 1977) which usually occurs on the extremities of young adults or children, and remains localised. Focal myositis may show evidence of denervation as well as a lymphocytic infiltration (Heffner and Barron, 1980).

The rash of dermatomyositis affects the face with a violaceous eruption in a butterfly distribution, involving the peri-orbital areas, spreading to the neck, upper chest and arms. The erythema may have a diffuse or mottled distribution, and red or dark lilac in colour. A lilac rash of the upper eyelids (heliotrope rash) is highly characteristic. Dusky red, scaly, elevated patches occur over the elbows, knuckles (Gottron's sign - which spares the phalanges in contrast to the rash of SLE), and to a lesser extent over the knees and medial malleoli. There is also hyperaemia at the base of the fingernails with red, shiny atrophic skin at the ends of the fingers. In very acute cases intradermal and subcutaneous oedema can occur.

Raynauds phenomenon occurs in about a quarter of cases, but is not usually severe unless part of progressive systemic sclerosis or mixed connective tissue disease (MCTD). Joint symptoms are common, with up to half having arthralgia at some stage (Pearson, 1958). Occasionally there may be joint effusions in a similar distribution to rheumatoid arthritis.

Parenchymal lung disease occurs in about 5% of cases, with a clinical picture of fibrosing alveolitis. Patients with lung involvement have a higher incidence of the anti-Jo-l antibody than cases without lung involvement (Yoshida *et al*, 1983).

Oesophageal involvement may cause reduced motility. In childhood dermatomyositis a vasculitis may cause gastrointestinal haemorrhage (Banker and Victor, 1966).

Thyroid disease may be more common in IIMD, in particular there appears to be an association between hypothyroidism and lung involvement (Miller *et al*, 1987).

The true incidence of cardiac involvement is hard to assess, but some clinical studies have suggested up to 100% involvement (Askari and Huettner, 1982), while others have found a lower incidence (Gottdeiner *et al*, 1978, Bitnum *et al*, 1964).

Involvement may produce a worse prognosis (Oka and Raasakka, 1978). The pathological changes found at autopsy usually consist of active myocarditis with myocardial necrosis and an inflammatory cell infiltrate, and are found in 30-50% (Haupt and Hutchins, 1982; Denbow *et al*, 1979; Banker and Victor, 1966). Some cases also show evidence of a vasculitis, particularly childhood cases and those with associated C-T disease (Haupt and Hutchins, 1982).

Table 2 - Summary of clinical findings in IIMD (Currie, 1981)

Symptom	Incidence
Muscular	
Weakness	
Shoulder girdle	75-100%
Pelvic girdle	75-100%
Distal muscles	30%
Neck flexors	65%
Dysphagia	50%
Facial muscles	rare
Respiratory muscles	rare
Pain or tenderness	50-75%
Contractures	<25%
Atrophy	50%
Skin	
Classical dermatomyositis	30%
Atypical rash	25%
Other	
Raynauds	20-30%
Arthralgia	30-50%
Gastrointestinal	rare
Pulmonary	5%
Cardiac symptoms	rare
Peripheral nerve	rare

1.3 Classification

Various classifications have been used, perhaps the most widely accepted is the following (Messner, 1988):

Type I Adult Polymyositis
Type II Dermatomyositis
Type III Juvenile Dermatomyositis
Type IV Myositis with malignancy
Type V Myositis with connective tissue disease

Recently a further sub-type, inclusion body myositis (IBM), has become recognised (Yunis and Samaha, 1971) with important differences in the clinical and pathological picture. This group constitutes about 25% of total cases of idiopathic inflammatory muscle disease with a male predominance and more common onset after the age of 50 years (Lotz *et al*, 1989). The presentation may be more insidious with both distal and proximal muscle involvement which is painless and associated with only mildly elevated serum CPK levels. Another important difference is that patients with IBM do not respond to immunosuppressive therapy (Lotz *et al*, 1989).

Perhaps a more useful classification would be:

Type I Polymyositis
Type II Dermatomyositis (a) Adult
(b) Juvenile
Type III Inclusion Body Myositis

All except IIb can be associated with connective tissue disease and types I and IIa may be associated with malignancy.

1.4 Associations

1.4.1 Malignancy

There has been much controversy over the possible association of IIMD and malignancy (Bohan and Peter, 1975). One large review suggested an incidence of 15% (Williams, 1959), however, most studies are biased because of the extensive search for malignancy carried out in patients with IIMD. A recent large study

(Sigurgiersson *et al*, 1992) found an increased incidence of malignancy in patients with IIMD, but more importantly an increased risk of mortality from malignancy in patients with dermatomyositis. This latter finding is less likely to be subject to bias, but a possible effect by steroid therapy on mortality in patients with malignancy cannot be excluded.

1.4.2 Connective tissue disease

20-30% of all cases have an associated connective tissue disease ("overlap syndrome"), in particular rheumatoid arthritis, Sjogren's syndrome, progressive systemic sclerosis, mixed connective tissue disease and systemic lupus erythematosus (SLE). Although these conditions may be associated with true myositis, muscle symptoms can be caused be disuse atrophy, denervation, steroid myopathy and vasculitis, which may also complicate connective tissue diseases. The true incidence of an inflammatory myopathy in each connective tissue disease is hard to quantify, in studies on SLE about 40% of those biopsied had evidence of a myositis (Oxenhandler *et al*, 1982; Isenberg and Snaith, 1981; Tsokos et al, 1981), but it is still not always easy to fully exclude the role of therapy (Eadie and Ferrier, 1966; Cucher and Goldman, 1976).

1.5 Investigations

1.5.1 Serum enzymes

Investigations usually demonstrate elevated serum levels of muscle enzymes, particularly creatine phosphokinase (CPK) and lactate dehydrogenase (LDH), reflecting muscle necrosis. High CPK is usually found in active disease and falls with remission, although it may only be raised in two thirds at presentation (De Vere and Bradley, 1975).

1.5.2 Electromyography (EMG)

EMG demonstrates a classical triad of:

1. Polyphasic, short, small motor-unit potentials on voluntary activity (90%).
2. Spontaneous activity, fibrillation, positive sharp waves with increased irritability (75%).
3. Bizarre, high-frequency, repetitive discharges evoked by mechanical stimulation of the muscle or movement of the electrode (40%).

Although these features are suggestive of IIMD they are not diagnostic (Marinacci, 1965). It should also be remembered that a neuropathic picture on EMG can be seen in IBM (Lotz *et al* 1989).

1.5.3 Muscle biopsy

Muscle biopsy findings are discussed below.

1.6 Diagnosis

Diagnosis can be difficult and in order to try and standardise diagnoses, particularly for the purposes of clinical study and treatment a set of diagnostic criteria have been suggested:

1. Symmetrical weakness of proximal muscles
2. Biopsy showing an inflammatory infiltrate
3. Elevated serum CPK
4. Myopathic EMG
5. Characteristic rash

For a definite diagnosis 4 or 5 of the above features are required; 3 for a probable diagnosis and 2 for a possible diagnosis (Bohan and Peter, 1975).

1.7 Autoantibodies

A number of circulating autoantibodies have been detected in patients with IIMD, some of which are seen in a number of other autoimmune conditions and others which appear to be specific to myositis (see Table 3).

Table 3 - Autoantibodies in IIMD (Plotz *et al*, 1989)

Antibody	% positive	Association
Non myositis specific		
Anti-nuclear antibodies	50-90	
Rheumatoid factor	10	
Anti-muscle	70-90	
Anti-thyroid	10	Autoimmune thyroid disease
Anti-nRNP	4-15	SLE
Anti-Ro/La	10-25	SLE and Sjogren's syndrome
Anti-PM/Scl	10	Systemic sclerosis
Anti-Ku	5	Systemic sclerosis
Myositis specific		
Anti-Mi-2	2*-21**	Dermatomyositis
Anti-Jo-1 (histidyl-tRNA synthetase)	10**-50*	Interstitial lung disease, arthritis, Raynauds disease.
Anti-PL-7 (threonyl-tRNA synthetase)	3-5	Interstitial lung disease
Anti-PL-12 (alanyl-tRNA synthetase)	<5	Interstitial lung disease
Anti-isoleucyl-tRNA synthetase	<5	Interstitial lung disease
Anti-glycyl-tRNA synthetase	<5	Interstitial lung disease
Anti-KJ	<5	Interstitial lung disease
Anti-tRNA	<5	Unknown
Anti-SRP (signal recognition peptide)	<5	Acute severe polymyositis

*polymyositis
**dermatomyositis

Many of the antibodies directed against muscle proteins, such as myosin or myoglobin, are also seen in other diseases where damage to muscle fibres occurs,

although higher titres are generally seen in myositis (Wada *et al*, 1983; Nishikai and Homma, 1977). Those restricted to IIMD are largely directed against cytoplasmic ribonuclear complexes, with the protein component being the antigen. The majority of these are aminoacyl-transfer RNA synthetases. It has been suggested that these enzymes may become antigenic following binding by viruses (Mathews and Bernstein, 1983; Bernstein and Mathews 1987). The most common is the anti-Jo-1 antibody, which is strongly associated with interstitial lung disease (Yoshida *et al*, 1983; Marguerie *et al*, 1990), and also is linked to HLA DR3 and DRw6 (Arnett *et al*, 1981). It is also much more common in polymyositis than dermatomyositis, and not found in childhood dermatomyositis (Cambridge, 1984).

Another antibody directed against a 56 kD nuclear ribonucleoprotein also appears to be myositis specific (Arad-Dann *et al*, 1987). These authors have found 85% of patients positive, having a stronger association with dermatomyositis (Arad-Dann *et al*, 1989).

The role for these antibodies is not known, and although fairly specific for IIMD they may be epiphenomena rather than of pathogenic importance. However, a better understanding of how they arise may be crucial to an understanding of the disease mechanisms.

1.8 Pathology

1.8.1 Histopathology

The different sub-groups show different patterns of histological changes within muscle, although all show varying degrees of inflammation, necrosis and regeneration of muscle fibres.

An early change in muscle fibres is alteration in the sarcoplasm, becoming more refractile and eosinophilic, with loss of striations. This may develop into hyalinisation or granular degeneration. These changes may affect segments of fibres (see Figure 25). Vacuolar change may also occur. Regenerating fibres have a lilac basophilia, with increased numbers of enlarged centrally located nuclei. Regenerating fibres, in particular the satellite cells have large amounts of cytoplasmic RNA. The inflammatory cell infiltrate may not parallel the changes in muscle fibres, and is usually patchy. The foci of mononuclear cells may be around vessels or endomysial. Most inflammatory cells are lymphocytes, which marker

studies have shown to be predominantly activated T lymphocytes (Rowe *et al*, 1981), with some macrophages, mast cells and plasma cells. If longstanding there may be prominent interstitial fibrosis.

In polymyositis the infiltrate is within fascicles and necrotic fibres are scattered rather than in groups, not always near to the inflammatory cell infiltrate.

In dermatomyositis the inflammatory infiltrate is perivascular or in septa and around fascicles with a relatively higher proportion of B lymphocytes than in polymyositis. Perifasicular atrophy is present in 90% of juvenile dermatomyositis (see Figure 1) cases and 50% adult dermatomyositis cases (Plotz *et al*, 1989). Also in dermatomyositis vascular changes are more prominent with muscle capillary depletion, and microtubular inclusions present within endothelial cells at electron microscopy (see Figure 52) (Emslie-Smith and Engel, 1990; Banker, 1975; De-Visser *et al*, 1989). The nature of these inclusions, which are also seen in skin endothelial cells, is not known, it has been suggested that they represent viral particles (Hashimoto *et al*, 1971), although they also resemble alterations of the endoplasmic reticulum (Baringer and Swoveland, 1972). Such inclusions may be non-specific, and can be seen in lymphocytes following stimulation with alpha-interferon (Grimley *et al*, 1985). Blood vessels in connective tissue, the gastrointestinal tract, fat and small nerves may also be affected, usually with a perivascular infiltrate. Endothelial hyperplasia with fibrin thrombi can occur causing micro-infarcts.

In dermatomyositis the pathological changes in the skin consists of oedema of the upper dermis with mucin deposition, vacuolation of the basal layer of the epidermis and sometimes a perivascular lymphocytic infiltrate. Endothelial swelling and vascular basement membrane thickening are particularly prominent in childhood dermatomyositis (Bowyer *et al*, 1986) and epidermal Langerhans cell abnormalities have been noted (Sontheimer and Bergstresser, 1982).

IBM is characterised by several histological features (see Figure 2) (Lotz *et al* 1989):

1. Single or multiple vacuoles rimmed with basophilic material
2. A predominantly endomysial mononuclear infiltrate
3. Invasion of non-necrotic muscle fibres by endomysial mononuclear cells
4. Groups of atrophic muscle fibres

5. Small refractile eosinophilic inclusions on haematoxylin and eosin stained sections

6. Tubulo-filamentous cytoplasmic and intranuclear inclusions 16-21 nm in diameter seen using electron microscopy (EM) (see Figure 50)

7. Membrane whorls within vacuoles at EM

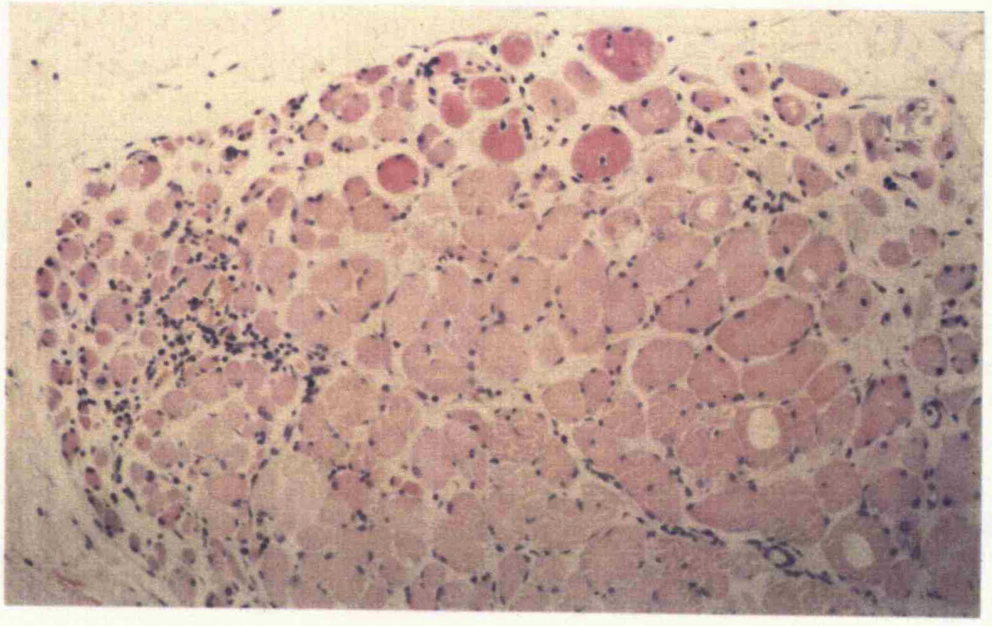


Figure 1. Haematoxylin and Eosin stained section from case 22 showing the characteristic perifasicular atrophy of juvenile dermatomyositis. Magnification X 52.5.

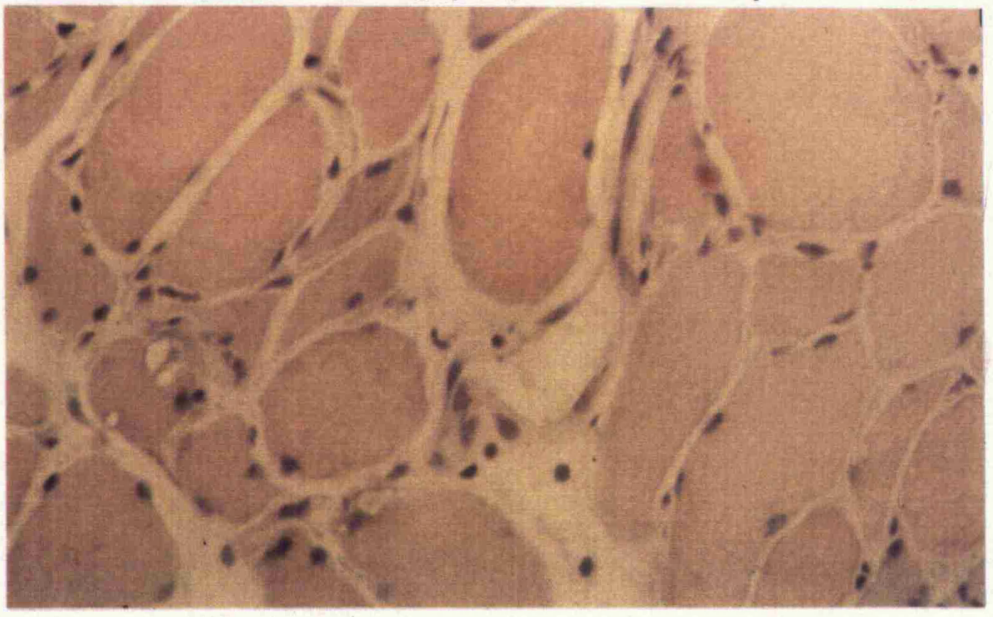


Figure 2. Haematoxylin and Eosin stained section from case 11 showing a "rimmed" vacuoles in several fibres and an eosinophilic inclusion typical of inclusion body myositis. Magnification X 210.

The nature of these highly characteristic filamentous inclusions is controversial. Similar inclusions, together with rimmed vacuoles, can also be seen in occulopharyngeal dystrophy and distal (Welander) myopathy (Lindberg *et al*, 1991). The inclusions were originally thought to resemble the nucleocapsids of paramyxoviruses (Chou, 1967) with a later study claiming that immunostaining for mumps virus antigen was positive in these cases (Chou, 1986). However, this finding has not been confirmed and two more recent studies using both immunocytochemistry and *in situ* hybridisation for the mumps virus were negative (Nishino *et al*; 1989; Kallajoki, 1991), apart from non-specific binding of antibody to the rim of the vacuoles in the earlier study (Nishino *et al*; 1989). It has also been shown that these filamentous structures are positive with congo red staining leading the authors to suggest that the disease may represent a form of prion disease (Mendell *et al*, 1991). One recent report has claimed to have demonstrated prion protein in these structures (Askanas *et al*, 1993). Another study has further characterised this amyloid by demonstrating β-amyloid immunoreactivity similar to that seen in Alzheimer's disease (Askanas *et al*, 1992).

The nature of the mononuclear inflammatory cell infiltrate in IBM does not appear to differ significantly from that of polymyositis (Figarella *et al*, 1990).

1.8.2 Immunopathology

In IIMD most infiltrating lymphocytes are activated T cells (Rowe *et al*; 1981, Rowe *et al*, 1983), but there are important differences in the distribution and proportions of various sub-types which is discussed below.

In dermatomyositis there is a higher percentage of B cells and increased ratio of CD4+ cells: CD8+ cells, with little invasion of non-necrotic fibres (Engel and Arahatak, 1986; Behan *et al*, 1987). This suggests a predominantly humoral process, possibly directed against the intramuscular vasculature which show the earliest signs of damage (De-Visser *et al*, 1989). Such vascular changes, with the formation of tubuloreticular inclusions in endothelial cells and a reduction in the number of vessel present, are not seen in polymyositis and IBM (Emslie-Smith and Engel, 1990). The degree of these changes is related to the amount of muscle damage (Casademont *et al*, 1990). Deposition of complement on vessels may be important in mediating this process, occurring early in the disease (Kissel *et al*, 1991), but is not specific to dermatomyositis (Whitaker and Engel, 1972). The

structural changes in muscle with necrosis, sometimes resembling areas of infarction, and perifasicular atrophy may be as a result of ischaemic damage.

In polymyositis and IBM there is no evidence of a microangiopathy and the attack is led by CD8+ lymphocytes, often around non-necrotic muscle fibres. The adjacent muscle fibres, unlike normal fibres, strongly express class I antigens on their sarcolemma (Karpati *et al*, 1988). Class I antigens are important in presentation of antigen to cytotoxic/suppresser (CD8+) lymphocytes. These antigens are also necessary for gamma-delta expressing lymphocytes mediate cytotoxicity; lymphocytes of this phenotype have also been demonstrated in IIMD, although rarely (Hohlfeld *et al*, 1991). However, increased class I antigen expression has also been demonstrated in fibres from cases of muscular dystrophy questioning the significance of this expression as an initiator of cell mediated attack (Isenberg *et al*, 1986; Appleyard *et al*, 1985). Others have demonstrated the expression of class II antigens on the sarcolemma in polymyositis, but not in other disorders in which there is an infiltrate within muscle (Zuk and Fletcher, 1988). Class II expression can be induced by treatment of human myoblasts with gamma-interferon (Mantegazza *et al*, 1991) which is known to be released in IIMD (Isenberg *et al*, 1986). Class II expression is necessary for presentation of antigen to CD4+ (helper/inducer) cells.

In view of the possible viral aetiology of IIMD it is of interest that viral infection of human cells can lead to class II expression (Fais *et al*, 1991; Khoury *et al*, 1991). One possible explanation of the cell mediated attack seen is that viral infection of muscle fibres leads to increased class II expression and possible presentation of viral or altered host antigens to the immune system. Mononuclear cells isolated from a case of dermatomyositis have been shown to be myocytotoxic, and this is HLA restricted in human muscle (Rosenschein *et al*, 1987).

1.9 Aetiological considerations

1.9.1 Genetic factors

IIMD rarely has a familial occurrence with occasional case reports of more than one member of a family being affected published (Lambie and Duff, 1963; Hennekam *et al*, 1990). There are also a few reports of IBM occurring in more than one family member, either in a dominant pattern (Neville *et al*, 1992; Klingman *et al*, 1991) or a recessive pattern (Cole *et al*, 1988; Massa *et al*, 1991). The rarity of such cases suggests that there is unlikely to be a major genetic cause in most cases.

In hereditary (X-linked) hypogammaglobulinaemia there is a form of encephalitis associated with myositis and a skin rash, resembling that of dermatomyositis (Webster *et al*, 1973; Ziegler and Penny, 1975; Wilfert *et al*, 1977; Mease *et al*, 1981). The condition may present insidiously with gradual progression over several years. Histological examination of the skin shows a mild pericapillary infiltrate of mononuclear cells, with a mononuclear infiltrate also present in skeletal muscle associated with muscle fibre atrophy (Webster, 1984). Echovirus can be cultured from the CSF and muscle in most of these cases, and is thought to be the aetiological agent.

1.9.2 Histocompatability studies

Several studies have suggested an association between certain HLA types and IIMD. HLA-B8 in particular has been associated with both childhood dermatomyositis (Packman *et al*, 1977), and adult polymyositis (Behan *et al*, 1978). Other associations have also been described (Hirsch *et al*, 1981; Cumming *et al*, 1978). The association with HLA-DR3 is interesting as in Caucasians this is linked with HLA-B8 in 96% cases. In the Negros population this linkage is uncommon, however, in those Negros with IIMD there is a 50% linkage (Plotz *et al*, 1989). In IBM there is a three fold increase in the DRl haplotype (50% compared with 15% controls). There may also be an association between B8-DR3 and the anti-Jo-l antibody (Plotz *et al*, 1989).

The linkage of IIMD to certain HLA phenotypes is in keeping with the hypothesis that IIMD is an autoimmune disease.

1.9.3 Drugs

A number of drugs and toxins have been implicated in the triggering of a few cases of IIMD. Several cases indistinguishable from IIMD have followed penicillamine ingestion (Morgan *et al*, 1981; Doyle *et al*, 1983; Schraeder *et al*, 1972; Ostensen *et al*, 1980; Cucher and Goldman, 1976; Essigman, 1982). The number of reported cases suggests a genuine association may exist. It has also been shown that the chances of developing myositis, compared with myasthenia gravis as a complication of penicillamine therapy, is related to HLA type, HLA-DRl being associated with myasthenia and HLA-DR4 with myositis (Garlepp and Dawkins, 1984). Occasional cases of IIMD have been linked to: cimetidine (Watson *et al*, 1983; Kaplusky *et al*,

1982); Tamoxifen (Harris *et al*, 1982); carbimazole (Pasquier *et al*, 1991); chloroquine (Eadie and Ferrier, 1966). The myopathy associated with azidothymidine (AZT) resembles a mitochondrial myopathy more than IIMD (Mhiri *et al*, 1991).

There have also been a number of cases of myalgia with eosinophilia after ingestion of contaminated tryptophan (Hertzman *et al*, 1990) and a few cases of IIMD associated with fish toxin (Stommel *et al*, 1991).

1.9.4 Epidemiological factors

Epidemiological studies have demonstrated seasonal variation in the presentation of cases of IIMD, with clustering of cases, particularly childhood cases, in the spring (Medsger *et al*, 1970; Manta *et al*, 1989). This seasonal variation in the occurrence of cases is suggestive of an infectious aetiology.

1.9.5 Viruses other than picornaviruses

A number of viruses have been proposed as aetiological agents in the pathogenesis of IIMD, perhaps the most likely are the picornaviruses which will be discussed later, the evidence for other viruses will be discussed here.

In one benign variant of myositis that follows a flu like illness in children with prominent calf pain lasting less than a week (Dietzman *et al*, 1976; McKinlay and Mitchell, 1976). This condition is associated with both influenza A or B infection (Dietzman *et al*, 1976; Ruff and Secrist, 1982). Histologically there is segmental muscle fibre necrosis early (Farrel *et al*, 1980) and a prominent inflammatory infiltrate within 5 days (Mejlszenkier *et al*, 1973). Influenza virus has also been linked to myositis with myoglobinuria (Gamboa *et al*, 1979).

Retroviruses can induce a myopathy with inflammation, necrosis and inclusions, in monkeys, and has been reported in patients with HIV infection (Dalakas *et al*, 1986a; Dalakas *et al*, 1986b). Following these reports many more cases of myositis have been reported in AIDS patients (Stern *et al*, 1987; Simpson and Bender, 1988; Wiley *et al*, 1989). The infiltrates in these cases show similarities to patients with IIMD (Illa *et al*, 1991) and HIV antigen has been demonstrated in infiltrating macrophages (Chad *et al*, 1990). However, it is not possible to be sure whether the myositis is due to therapy, HIV or an opportunistic infection.

A few other cases of IIMD have been linked to hepatitis B (Mihas *et al*, 1978; Pittsley *et al*, 1978; Damjanov *et al*, 1980) and herpes zoster infection (Norris *et al*, 1969).

1.9.6 Toxoplasma gondii

One of the first reports of toxoplasma infection causing extensive muscle damage occurred in a 60 year old woman, who at post-mortem had necrosis, regeneration and an inflammatory cell infiltrate in skeletal muscle with toxoplasma organisms present (Kass *et al*, 1952).

Since then there have been many cases of IIMD associated with serological or morphological evidence of toxoplasma infection reported (Rowland and Greer, 1961; Chander *et al*, 1968; McNicholl and Underhill, 1970; Greenlee *et al*, 1975; Samuels and Reitschel, 1976; Hendrickx *et al*, 1979; Pollock, 1979; Topi *et al*, 1979; Schroter *et al*, 1987; Yamada *et al*, 1989). Most had proximal muscle weakness, elevated CPK, abnormal EMGs and 4 had a heliotrope rash. Muscle biopsies showed inflammation with organisms seen directly in only 3 cases (Chander *et al*, 1968; Greenlee *et al*, 1975; Topi *et al*, 1979), and in a further case with the aid of direct immunofluorescence. Some of these cases showed clinical response to antiprotozoal therapy (Chander *et al*, 1968; Samuels and Reitschel, 1976; Pollock, 1979; Schroter *et al*, 1987).

There have also been a number of studies looking for serological evidence of toxoplasma in cases of polymyositis:

(i) Kagen *et al* (1983) reported 8/10 patients with polymyositis had positive Sabin-Feldman dye tests, 6 with titres of 1:1024 or more. Also 4/11 patients with dermatomyositis had positive tests, 3 with titres of 1:1024 or greater. High titres were associated with recent onset (less than 1 year) disease.

(ii) Phillips *et al* (1979) found increased anti-toxoplasma IgM antibodies in cases of polymyositis but not cases of dermatomyositis, myositis associated with connective tissue disease or a large number of controls.

(iii) Magin and Kagen (1983) found that 50% of 58 patients with either polymyositis or dermatomyositis had positive Sabin-Feldman dye tests and 24%

raised anti-toxoplasma IgM antibodies by immunofluorescence. None of 30 patients with dystrophy and only 3% of 58 patients with SLE (including 8 with myositis) had anti-toxoplasma IgM antibodies. The absence of anti-toxoplasma IgM antibodies in other types of muscle disease suggests that this response is not simply due to reactivation of dormant organisms in damaged muscle.

It has been suggested that the toxoplasma infection is secondary to impaired immune response in polymyositis patients (Behan *et al*, 1983), however studies on mice has shown that inoculation with toxoplasma causes a myositis in 44% of cases (Henry and Beverley, 1969). The mice develop a focal myositis with muscle fibre necrosis, oedema and inflammation, although no free trophozoites were seen in these areas.

There are a number of possible explanations for the association of toxoplasma gondii antibodies and IIMD:

(i) Coincidental infection due to host immune dysfunction or secondary to immunosuppressive treatment.
(ii) Reactivation of dormant organisms.
(iii) Non-specific activation of the immune system.
(iv) Infection causes IIMD.

No formal histopathological study has been carried out to investigate these possibilities further.

1.10 Treatment

The main line of treatment consists of various forms of immunosuppression, particularly steroid therapy. This form of therapy is widely used and although no large controlled studies have been carried out, its efficacy is generally accepted. IBM remains resistant to this form of treatment (Lotz *et al*, 1989).

Usually several weeks of high dose (e.g. 1 mg/kg body weight) prednisolone is recommended, followed by a gradual reduction. Response should be measured by an objective measure of muscle strength, rather than a feeling of increased energy or a reduction of serum creatine phosphokinase. If there is no response to steroids after 3 months then treatment should be changed to another form of immunosuppression such as azathioprine and methotrexate. Cyclophosphamide, cyclosporin, plasmaphoresis and chlorambucil have also been used. Total-body irradiation and intravenous immunoglobulin can also be effective (see review: Dalakas, 1991).

1.11 Prognosis

About 50% of patients show a prolonged remission with steroid therapy (Currie, 1981). Those cases with associated lung involvement, connective tissue disease or malignancy have a worse outlook (De Vere and Bradley, 1975) and IBM follows a relentless course despite treatment.

2. GENERAL FEATURES OF THE PICORNAVIRIDAE

2.1 Introduction

Picornaviruses are a family of small RNA viruses which cause a number of diseases in humans and animals. They are have caused human disease for thousands of years, with cases of paralytic polio recorded in the l4th century B.C. by Egyptian art. There are over 200 serotypes which are classified into four genera. The enteroviruses are the most important in terms of human pathogenicity, causing 5-10 million symptomatic infections annually in the United States (Strikas *et al*, 1986), and include the polioviruses, coxsackieviruses and echoviruses. Rhinoviruses are the major cause of the common cold. Foot-and-mouth disease virus are also of great importance in farming, being the commonest single pathogen.

Table 4 - Picornaviridae (Rueckert, 1990)

Genus	Members	Numbers of serotypes
Enterovirus	Human Poliovirus	3
	Human Coxsackievirus A	23
	Human Coxsackievirus B	6
	Human Echovirus	32
	Human Hepatitis virus A	1
	Thielers Murine Encephalomyelitis virus	1
	Human Enterovirus	4
	Bovine Enterovirus	2
	Simian Enterovirus	18
	Porcine Enterovirus	11
	Vilyunsk virus	1
Rhinovirus	Human Rhinovirus	10
	Bovine Rhinovirus	2
Cardiovirus	Encephalomyocarditis virus	1
Aphthovirus	Foot-and-mouth disease virus	7
Unassigned	Equine rhinoviruses	2
	Cricket paralysis virus	1
	Drosophilia C virus	1
	Tussock moth picornavirus	1

These viruses share a number of characteristics including size, icosahedral morphology, lack of lipid envelope, a positive stranded RNA genome and the general arrangement of their structural proteins

2.2 Classification

The picornavirus family is divided into four genera: the enteroviruses, the cardioviruses, the rhinoviruses and the apthoviruses. None can be distinguished on electron microscopy.

Enteroviruses inhabit the alimentary tract, whereas the rhinoviruses are adapted to the nasopharyngeal region. Apthoviruses infect cloven-footed animals (cattle, goats, pigs, sheep) and rarely humans. Cardioviruses are predominantly murine viruses.

The enteroviruses, cardioviruses, Thieler's viruses and hepatitis A viruses are acid stable, surviving pH 3 or less, allowing passage through the stomach, while the rhinoviruses and apthoviruses are labile at < pH 6.

2.3 General structure

2.3.1 Capsid

Picornaviruses are non-enveloped and have a 25-30 nm capsid of icosahedral symmetry made up of 60 copies of the four capsid proteins: VP1, VP2, VP3 and VP4 (Rueckert, 1990). This capsid has several important functions:

(i) It protects the RNA genome from nucleases in the environment.
(ii) It recognises specific cell-coded receptors in the plasma membrane, which also determines host range and tissue tropism's.
(iii) The protomer determines antigenicity, carries directions for genome selection/packaging and provides a proteinase for maturation.
(iv) The capsid is designed to release the RNA genome into the cytoplasm of susceptible host cells.

2.3.2 Genome

The capsid proteins surround a single stranded, positive sense RNA genome of 7200 - 8500 nucleotides. The genome can be infective, but demonstrates extreme

sensitivity to ribonuclease once separated from its protein capsid. Sequencing of the genomes of several picornaviruses has revealed a common structure, the 5'-terminal nucleotide of which is covalently linked to a small virus encoded protein, VPg. The genome contains a 5' untranslated region (5' UTR) varying in length from 600 (rhinoviruses) to 1250 (apthoviruses) nucleotides, followed by an open reading frame (ORF) in excess of 2000 codons and a 3' UTR and a poly(A) tract. The 5' UTRs of cardioviruses and apthoviruses contain a long poly(C) tract, between VPg and the beginning of the protein-coding region, which is absent in the enteroviruses and rhinoviruses. Cardioviruses and apthoviruses (together with Theiler's murine encephalomyelitis virus (TMEV) are further characterised by a leader protein encoded prior to the capsid proteins.

2.3.3 5' untranslated region

The 5'UTRs of picornaviruses are long compared to those of many cellular and viral RNAs comprising about 10% of the total genome with many highly conserved regions. There are a few major differences such as an insertion of 100-140 nucleotides at about position 600, present in enteroviruses but not rhinoviruses. The significance of the unusual poly(C) tract in the 5'UTR found in cardioviruses and apthoviruses, is unclear.

The base composition of the 5'UTR is different to the rest of the genome, being higher in GC content. This probably reflects the complex secondary structure in this region for which there are several predicted models (Rivera at al, 1988; Skinner *et al*, 1989; Pilepenko *et al*, 1989). The predicted structural domains located at positions 236 - 443, 451 - 559 and 581 - 620 (sequence numbers from poliovirus type 3) is supported by the retention of this structure despite variations in nucleotide sequence ("covariance"). This secondary probably has important functions in both translation and neurovirulence (Skinner at al, 1989; Pilepenko *et al*, 1989; Nicholson *et al*, 1991).

The functions of the 5'UTR are not fully understood, but it is almost certainly involved in ribosomal binding to internal sequences known as the ribosome landing pad (RLP), the sequences of which have been investigated in poliovirus (Nicholson *et al*, 1991). This method of ribosomal interaction differs from that of most cellular mRNAs which use the 'scanning' model (Kozak, 1989) in which the ribosome initiates translation after 'scanning' along from the 5' terminus to a start sequence (AUG). In this situation it may be important to have a consensus sequence of A/G

at -3 and G at +4 from the initiation codon. In rhinoviruses the RLP may be closely followed by the initiation codon, whereas in enteroviruses there are an extra 100-140 bases from the proposed RLP to the AUG codon (Stanway, 1990).

This method of viral translation is important as cap-dependant mechanisms are suppressed during infection inhibiting cellular functions and allowing preferential synthesis of viral proteins.

2.3.4 Capsid proteins

Four capsid proteins, VP 1-4, are coded for at the 5' end of the open reading frame. VP 1-3 are all of similar size (m.w 26-33,000), VP 4 being much smaller (m.w. 7,000) in sizes (Kitamura *et al*, 1981). VP 1-3 are arranged in an 8 stranded β-barrel. The similarity of the folding mechanism for these three proteins suggests that they evolved from a common gene, despite differences in amino acid sequences.

The basic building block of the icosahedral capsid is a pentamer made up of five copies of VP 1-4, with VP 4 on the internal side. The pentamer is stabilised by interactions involving the N and C termini of VP 1 and 3 together with VP 4. Adjacent pentamers are held together by hydrogen bonds between VP 2 and VP 3. The relative weakness of these interactions may be important in the uncoating of viruses on entry into cells, which may be aided by phosphorylation of capsid proteins (Ratka *et al*, 1989).

Capsid assembly is via pentameric intermediates composed of copies of VPl, VP3 and the precursor protein VP0 (VP2 covalently linked to VP4). Cleavage of VP0 is one of the final steps in capsid assembly and may be related to encapsidation of the nucleic acid and to stabilisation of the mature particle. Picornavirus proteins are non-glycosylated, but VP4 is modified at its N terminus by a linkage to myristic acid, which may be important in capsid assembly (Chow *et al*, 1987). The potential for VP2 and VP4 to be phosphorylated by cellular kinases, thereby destabilising the capsid, may play a role in uncoating of the nucleic acid (Ratka *et al*, 1989).

Picornaviruses have a deep canyon, lined by VP 1 and 3, and it is thought that sites for interaction with virus-receptors may lie within this canyon. A short peptide sequence, RGD (Arg-Gly-Asp), which is known to inhibit attachment of some picornaviruses (Fox *et al*, 1989) has been identified in the VP 1 protein of coxsackie

A9 (Chang *et al*, 1989). This peptide may therefore be important in picornavirus attachment to cells.

However, not all picornaviruses share the same receptor. The best characterised is rhinovirus binding to the adhesion molecule, ICAM-1, found on many cells (Greve *et al*, 1989). Members of the coxsackie B group recognise a different receptor which appears to be a 50 kD membrane protein (Hsu *et al*, 1988).

2.3.5 Non-structural proteins

There are 7 basic non-structural proteins: 2A, 2B, 2C, 3A, 3B, 3C and 3D. In general these proteins are better conserved between picornavirus strains than the capsid proteins.

The most conserved protein is 3D, an RNA dependant RNA polymerase. Protein 2C which is thought to be involved in RNA replication, is also highly conserved.

Once the giant polyprotein is synthesised in translation it is cleaved by a series of protease steps, mainly involving protein 3C, a cysteine proteinase. Another protease, 2A, cleaves at the P1-P2 junction (i.e. at its own N terminus), separating the capsid proteins from the non-structural proteins.

The cleavage of VP0 to VP4 and VP2 is the last step in maturation of the virus particle and is thought to occur autocatalytically. VPg is a small protein covalently linked to the 5' terminus of all newly synthesised RNAs, of both positive and negative sense. Removal of VPg appears to be important in directing viral RNA to ribosomes, so favouring protein synthesis early in the infection cycle (Ruerckert, 1990).

The roles of non-structural proteins 2B and 3A are not understood.

2.3.6 3' untranslated region

The 3 'UTR differs in length between 40 nucleotides in rhinoviruses and 126 nucleotides in EMC virus, and is generally AU rich. Enteroviruses can be divided into two groups on the basis of this region, a poliovirus group (including coxsackie A21 and bovine enterovirus) with approximately 72 residues, and a coxsackie B group (which includes swine vesicular disease virus, coxsackie A9 and Echovirus

types 6 and 11) with about 100 residues. The extra bases correspond to a complete stem-loop structure predicted in coxsackie B viruses (Auvinen *et al*, 1989).

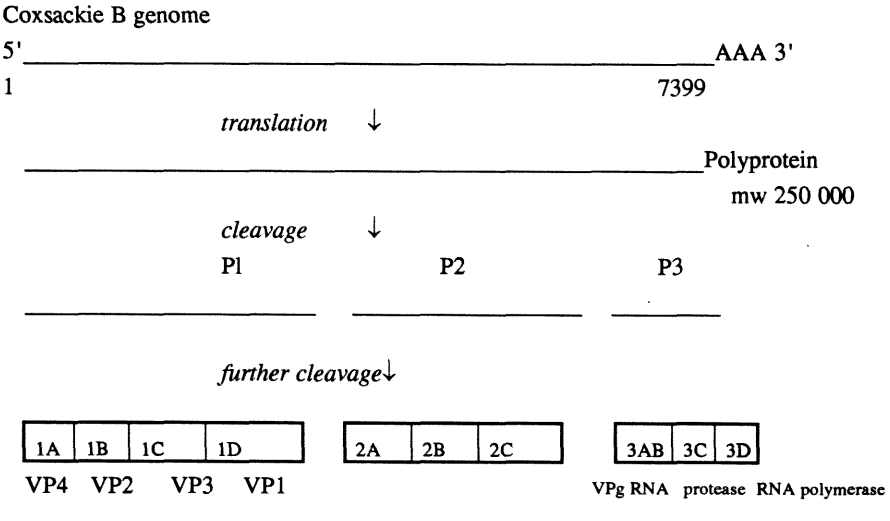
The 3' UTR is polyadenylated, with 35 residues in cardioviruses and 100 in apthoviruses.

2.4 Replication cycle

The initial event is attachment of virions to receptors on the plasma membrane which leads to release of viral RNA into the cytoplasm by a poorly understood mechanism.

Once in the cytoplasm the RNA acts as messenger RNA and is translated by host ribosomes, forming "polyribosomes", to form a single giant polyprotein. This is cleaved proteolytically to form a coat precursor protein (Pl), a mid-piece protein (P2) and a right piece protein (P3). P3 cleaves autocatalytically to form three smaller proteins: 3C - a proteinase which causes further cleaving of viral proteins; 3AB - a protein which gives rise to VPg, important in RNA synthesis; and 3D - a RNA dependant RNA polymerase which copies the +ve strand to form a complimentary -ve strand with poly(U) at it's 5' end (see below).

Diagram to illustrate coxsackie virus replication



Further synthesis of +ve RNA occurs on the -ve strand, which becomes multi-stranded, and is known as the replicative intermediate (RI). The RI is associated with the smooth endoplasmic reticulum, and generates a large excess of +ve RNA for translation. As the concentration of protein increases +ve stranded RNA is packaged into virions.

RNA synthesis proceeds exponentially, the rate doubling every 15 minute until 10% of the final yield is reached, when the rate become constant. Normally 5-10% of the total RNA is -ve stranded (Baltimore and Girard, 1966).

2.5 Effects on the host cell

Much work has been done on cytolytic viruses such as poliovirus, but little is known about persistent infections such as with Hepatitis A.

Characteristic morphological changes occur with many picornaviruses, with margination of nuclear chromatin occurring after about 1 hour, and cytoplasmic vesicles after 2-3 hours (Dales *et al*, 1965). At this stage the cell membrane becomes leaky and many viral crystals can be seen in the cytoplasm. These cytopathic effects may be due to redistribution of lysosomal enzymes, rather than changes in cellular DNA, RNA and protein synthesis (Rice and Wolff, 1975).

Cellular ribosomal and mRNA synthesis declines soon after infection, with strong inhibition of polymerase III, possibly mediated by a viral protein (Baltimore *et al*, 1963). An important factor in the shutoff of host protein synthesis, is the inactivation of translational initiation factor (eIF-4F or cap-binding complex) (Etchson *et al*, 1984). Binding of this particle by most cellular mRNAs is essential for translation by ribosomes (Kozak, 1989).

Infection halts cell division and DNA synthesis, but these effects are probably secondary to cessation of RNA and protein synthesis.

2.6 Evolution

The evolution of genetically diverse picornaviruses is due to two main mechanisms: the accumulation of uncorrected mutations resulting from errors in RNA replication, and RNA recombination. RNA polymerases do not use a DNA template to prime their action and their lack of exonuclease activity leads to a high mutation rate of

about 1 base/genome (Smith and Inglis, 1987), creating a very heterogeneous population and leading to rapid evolution.

Recombination may occur between related viruses, for example the sequence of coxsackie A21 (Hughes *et al*, 1989) would suggest that this is a recombinant with the 3' terminal 2000 nucleotides have been donated by poliovirus. The rate of recombination is very high, in one study a normal child given trivalent polio vaccine developed two different recombinants within a few weeks (Minor *et al*, 1986). It has also been estimated that 10-20% of all viral RNA molecules produced in a single growth cycle are recombinant (King, 1988).

Another possible mechanism of evolution is the introduction of host RNA into the viral genome. A togavirus has recently been shown to contain an inserted sequence encoding animal ubiquiten (Meyers *et al*, 1989).

3. COXSACKIE VIRUSES

3.1 Physical properties

Like other enteroviruses, the coxsackie viruses are acid stable, surviving pH 3 or less, this is in contrast to rhinoviruses which replicate in the nasopharynx and do not have to survive passage through the stomach. However, they are thermolabile and are destroyed rapidly at temperatures of 50°C. They are stable frozen for many years. Ultraviolet light and drying also inactivates them.

Many laboratory disinfectants are ineffective, including 70% alcohol, 1% Lysol and 1% quaternary ammonium agents, but they are inactivated by 0.3% formaldehyde, 0.1 N HCl or free residual chlorine at 0.3-0.5 ppm.

They are resistant to all known antibiotics and conventional chemotheraputic agents.

3.2 Epidemiology

Humans are the only known reservoir for human enteroviruses, and close human contact is the main route of spread. House flies can act as carriers, but the virus does not appear to multiply within them (Melnick and Penner, 1952). Most cases of coxsackie virus infection occur in childhood, and there is a marked seasonal variation with a peak of cases in August (Moore, 1982). In the UK there may be a cyclical pattern to the number of reported cases, especially of coxsackie B2-5 (Melnick, 1990). As with other enterovirus infections there is a male predominance. The most frequently reported types for coxsackie B are 5 and 3, and for coxsackie A types 9 and 16 (Grist *et al*, 1978).

3.3 Pathogenesis

The portal of entry is thought to be the alimentary tract via the mouth, with an incubation period of 7-14 days. After initial multiplication possibly in gut lymphoid tissue viraemia occurs with proliferation in the cells of the reticuloendothelial system and target organs.

The clinical syndromes caused will be discussed separately:

3.3.1 Asymptomatic infection

A high rate of asymptomatic infection is seen in all enteroviruses, with 76% of coxsackie virus infections being sub-clinical (Minor and Bell, 1990).

3.3.2 Meningitis

So called "aseptic" meningitis is most commonly caused by echoviruses, but a number of coxsackie viruses, especially types A9, B1-5 can cause outbreaks (Grist *et al*, 1978).

The prognosis is good, and there is usually a lymphocytic infiltrate in the cerebrospinal fluid.

3.3.3 Paralysis

The first report of coxsackie virus infection was in children with paralysis (Dalldorf and Sickles, 1948); since then many strains have been associated with individual case reports of paralysis and coxsackie A7 with outbreaks (Grist, 1962).

There is also some serological evidence to link coxsackie viruses with motor neuron disease (Kennedy *et al*, 1988; Bartfeld *et al*, 1989), raising the possibility that persistent viral infection of motor neurons can cause chronic neurological deterioration.

3.3.4 Encephalitis

Encephalitis is a rare complication of enterovirus infections, and coxsackie B virus has been demonstrated both serologically and by *in situ* hybridisation, with signal present around blood vessels and in the meninges, in one fatal case (Hallam, 1986).

3.3.5 Gastrointestinal infection

Although coxsackie viruses can sometimes cause gastrointestinal upset, they are a relatively unimportant cause of diarrhoea.

3.3.6 Hepatitis

A severe necrotising hepatitis can be seen in generalised perinatal coxsackie virus infection (Lake *et al*, 1976; Kaplan *et al*, 1983), but it is probably not a cause of hepatitis in older children and adults.

3.3.7 Pancreatitis and diabetes mellitus

Children with coxsackie virus infection have pancreatic lesions and rising titres to coxsackie viruses have been demonstrated in adults with pancreatitis (Capner *et al*, 1975). It is well recognised that mice infected with coxsackie viruses become hyperglycaemic with definite tropism's for pancreatic β-cells (Toniolo *et al*, 1982). Raised antibodies to coxsackie B viruses has been found in children with type 1 diabetes (King *et al*, 1983) and virus has been isolated from a child with diabetic ketoacidosis (Yoon *et al*, 1979). Attempts to demonstrate persistence of coxsackie RNA in tissue from the pancreases of patients with type 2 diabetes have failed (Lohr and Oldstone, 1990).

3.3.8 Myocarditis and dilated cardiomyopathy

Evidence for a direct association between coxsackie viruses and cardiac disease is very strong; raised antibody titres to coxsackie viruses has been found in patients (Muir *et al*, 1989; El-Hagrassy *et al*, 1980), there is a well established murine model of myocarditis induced by coxsackie viruses (Reyes *et al*, 1981; Estrin and Huber, 1987), and enteroviral RNA has been demonstrated in tissue from human myocardium in both myocarditis and dilated cardiomyopathy by several different methods (Bowles *et al*, 1986; Bowles *et al*, 1989; Kandolf *et al*, 1987; Easton and Eglin, 1988; Jin *et al*, 1990; Weiss *et al*, 1991).

3.3.9 Idiopathic inflammatory muscle disease and epidemic myalgia (Bornholm disease)

This will be discussed in section 4.

3.3.10 Respiratory disease

A number of coxsackie viruses have been associated with mild upper and lower respiratory tract infections (Dalldorf and Melnick, 1965).

3.3.11 Eye disease

Epidemics of mild-severe conjunctivitis have been linked to coxsackie A24, the virus being isolated from conjunctival swabs (Mirkovic *et al*, 1974). Recovery is usual after 1-2 weeks.

3.3.12 Herpangina

This is characterised by fever, sore throat, pharyngeal vesicles sometimes with anorexia, dysphagia, vomiting and abdominal pain, occurring in children. It is mainly associated with coxsackie A viruses (Grist *et al*, 1978).

3.3.13 Hand, foot and mouth disease

A vesicular rash on the buccal mucosa, hands and feet can be caused by coxsackie viruses, in particular coxsackie Al6 (Cherry and Jahn, 1966).

3.3.14 Myalgic encephalomyelitis (post-viral fatigue syndrome)

This is a poorly defined condition, characterised by depression and persistent muscle fatigue following a minor viral illness (Holmes *et al*, 1988). Several studies have claimed an association between this condition and coxsackie viruses (Yousef *et al*, 1988; Dowsett *et al*, 1990). Also persistent enterovirus RNA has been demonstrated in skeletal muscle from cases (Archard *et al*, 1988; Cunningham *et al*, 1990). However, other studies have failed to confirm an association (Miller *et al*, 1991) and one group having sequenced the amplified nucleic acid raise the possibility that it could also be calsequestrin mRNA, a normal muscle product (Gow and Behan, 1991).

3.3.15 Congenital abnormalities

Congenital cardiac abnormalities and rare CNS malformations have been associated with both maternal and infant coxsackie infection (Gauntt *et al*, 1985; Brown and Evans, 1967; Brown and Karunas, 1972).

There is also evidence that endomyocardial fibroelastosis is a result of coxsackie B infection, with 13/28 cases having evidence of infection (Fruhling *et al*, 1962).

3.3.16 Neonatal infections

Severe, often fatal infections occur in infants. Three patterns have been identified in a review of 41 cases (Kaplan *et al*, 1983):

1. Explosive and fulminant viraemic illness with death within 12 hours.
2. A biphasic illness, starting with mild symptoms followed by a severe myocarditis after about 10 days.
3. A progressive illness starting soon after birth terminating with myocarditis, vascular collapse and/or a bleeding diathesis.

Most mothers had minor symptomatic viral illnesses soon before or after birth, and it is assumed infant infection occurred either transplacentally or via the birth canal.

3.4 Diagnosis

Enteroviruses are ubiquitous and can be isolated from up to 50% of healthy day-nursery children, even in non-epidemic periods (Grist *et al*, 1972). In view of this it is important to fulfil a number of criteria before ascribing an illness to any of these viruses:

(a) There is a higher percentage of patients infected with virus than healthy individuals of the same age, social class and living in the same area.
(b) Antibodies for the virus develop during the course of the disease, and serological evidence is negative for other agents also known to cause the clinical syndrome.
(c) The virus is isolated from body tissues or fluids affected by the disease.

3.5 Laboratory culture

Coxsackie viruses are found in the stools, where they may be excreted for weeks following an infection; rectal or throat swabs; cerebrospinal, conjunctival or pericardial fluid.

Originally they were identified by their ability to infect newborn mice (Dalldorf and Sickles, 1948), this method is still used, particularly for the coxsackie A viruses, which can be difficult to grow in cell lines. Classically coxsackie A infected mice develop a widespread myositis and flaccid paralysis, while those infected with coxsackie B develop a more focal myositis, encephalitis and fat necrosis, with a spastic paralysis (Minor and Bell, 1990). A detailed account of the pathological changes produced has been described (Roberts and Boyd, 1987). Unlike the polio viruses, coxsackie viruses do not usually cause symptomatic infection in chimpanzees or cynomologous monkeys, although types A7 and A14 can induce paralysis (Dalldorf, 1957; Voroshilova and Chumakov, 1959).

In most instances coxsackie viruses do not infect adult mice, however coxsackie A viruses can infect denervated mature skeletal muscle or muscles in which acetylcholine release has been blocked by botulinium toxin (Andrew *et al*, 1984). Group B coxsackie viruses also infect adult mice if they have suffered undernutrition, when a severe chronic viral infection is produced which may be reversed by transfer of lymphoid tissue from normal mice (Woodruff and Woodruff, 1971).

A number of cell lines can be used to demonstrate the cytopathic effect of coxsackie viruses; the RD cell line derived from a human rhabdomyosarcoma (McAllister *et al*, 1969) is particularly good for coxsackie A strains, although types A1, A19 and A22 still need newborn mice, another cell line derived from buffalo green monkey kidney cells is also useful for both groups, particularly group B strains (Menegus and Hollick, 1982). The cytopathic effect is characterised by cells shrinkage, nuclear pyknosis, becoming refractile and eventually falling off the glass surface.

For most strains specific identification of a serotype rests with serum neutralisation testing using internationally standardised hyper immune sera (Melnick *et al*, 1973), which can be used as 8 pools to simplify identification.

3.6 Antibody tests

Paired specimens, taken early in the disease and 3-4 weeks later are used. The neutralisation test is accurate and type specific. Acute and convalescent sera are serially diluted and tested against a constant amount of specific virus. A fourfold or greater rise in antibody titre is considered indicative of recent infection (Melnick, 1990). If neutralising titres are high at the start of infection interpretation may be difficult, as titres can remain for years after an initial infection. Another problem is the appearance of antibodies to other enteroviruses at the time of infection. Measurement of specific viral antibodies to a pan-coxsackie B epitope in the IgM fraction is useful in determining recent infection as these antibodies only persist for 6-8 weeks (Dorries and ter Meulin, 1983).

Compliment fixing antibodies are variable and not useful for type specificity, except for the first enterovirus infection. After a coxsackie virus infection compliment fixing antibodies may develop to a number of group A and B strains, as well as echoviruses (Kraft and Melnick, 1952).

3.7 Detecting viral antigens

A rapid and sensitive enzyme-linked immunosorbent assay (ELISA) has been developed for the coxsackie viruses (Yolken and Torsch, 1981). This method can correctly identify all serotypes of coxsackie B viruses and 22 out of 23 coxsackie A viruses. However, this method has not been widely used, largely due to the expense (Minor and Bell, 1990).

An enterovirus group specific monoclonal antibody to VP1 epitopes has also been described, which reacts with coxsackie A and B, polio and echo viruses does not cross react with hepatitis A (Yousef *et al*, 1987).

3.8 Hybridisation studies

Numerous studies have now been published using various hybridisation methods to identify enteroviruses (see reviews: Hyypia, 1989 and Rotbart, 1991). Group specific probes have been developed which detect a wide range of enteroviruses (Tracy, 1985; Hyypia *et al*, 1984) as well as the more sensitive polymerase chain reaction (Chapman *et al*, 1990; Rotbart, 1990; Olive *et al*, 1990; Hyypia *et al*,

1989). Although these methods have the advantage of being rapid and highly sensitive, they are not serotype specific and can be expensive.

Perhaps the main use of these techniques is in research in order to better understand relationships between viruses and disease. Using *in situ* hybridisation viral RNA has been demonstrated in myocardial cells in myocarditis and dilated cardiomyopathy (Kandolf *et al*, 1987; Easton and Eglin, 1988; Bowles *et al*, 1986). Similar studies suggesting the presence of enteroviral RNA in skeletal muscle from patients with IIMD have been discussed elsewhere.

3.9 Treatment

At the moment no specific antiviral therapy is available for use in human picornavirus infection, however there are a number of drugs which appear effective in animal studies (Jubelt *et al*, 1989; McKinlay *et al*, 1986; McSharry *et al*, 1979).

4. EVIDENCE LINKING PICORNAVIRUSES TO THE AETIOLOGY OF IIMD

There are several lines of evidence suggesting a picornavirus aetiology, and in particular coxsackie virus, for IIMD. These will be discussed separately under the following headings:

(i). Myotropism
(ii). Molecular mimicry
(iii). Morphological evidence
(iv). Serology
(v). Virus isolation
(vi). Detection of viral RNA
(vii). Murine models
(viii). Other evidence

4.1 Myotropism

Coxsackie viruses demonstrate clear myotropism in both humans and animals. The first isolation of coxsackie viruses in children with paralysis demonstrated infection of suckling mice and hamsters, which produced severe muscle damage (Dalldorf and Sickles, 1948). The distribution of the changes has been further characterised with a diffuse pattern of muscle damage occurring with coxsackie A viruses and a more focal pattern with coxsackie B viruses (Roberts and Boyd, 1987). Outbreaks of epidemic myalgia (Bornholm disease) have been reported, which causes a self limiting condition with severe chest pain and fever (Sylvest, 1934) and evidence of inflammation in muscle specimens (Lepine *et al*, 1952), has been strongly associated with coxsackie B infection, especially coxsackie B5 (Weller *et al*, 1950).

4.2 Molecular mimicry

Antibodies to transfer RNA synthetases are the hallmark of IIMD, the commonest found anti-Jo-1 is directed against histidyl-tRNA synthetase (Mathews and Bernstein, 1983). It has been suggested that it may be caused by interactions between viral RNA, possibly complexed with histidine and the synthetase, to overcome immunological tolerance in some way to cause the production of autoantibodies (Walker and Jeffrey, 1986). An alternative explanation is mimicry of a viral epitope by a cellular protein, such sequence homology between the encephalomyocarditis

virus VP1 protein and histidyl-tRNA synthetase has also been reported (Walker and Jeffrey, 1988).

4.3 Morphological evidence

Coxsackie virus like particles were first seen at electron microscopy in a case of dermatomyositis (Zweymuller, 1953). Since then there have been numerous other cases reported, where crystalline arrays, said to resemble picornavirus particles (Gyorky *et al*, 1987; Chou and Gutman, 1970; Mastaglia and Walton, 1970; Ben-Bassat and Machtey, 1972; Tang *et al*, 1975).

However, in a case of polymyositis with similar particles it was shown that the particles showed staining typical for glycogen (Katsuragi *et al*, 1981), which is known to accumulate in polymyositis (Carpenter and Karpati, 1984).

Similar particles have also been seen in skeletal muscle from a number of other conditions including scoliosis (Webb and Gillespie, 1976); malignant hyperthermia (Schiller and Mair, 1974); normal extra-occular muscles (Caulfield *et al*, 1968) and normal muscles (Fukuhara, 1979). Also a case of heat stroke has been reported in which picornavirus-like particles were present in skeletal muscle (Burch *et al*, 1968), although the clinical details given in this case were rather sketchy.

4.4 Serology

Surprisingly few serological studies have been published, in the first, four consecutive adult cases of IIMD showed elevated neutralising antibodies to coxsackie B1, B3 and B4 providing evidence of recent coxsackie B virus infection (Travers *et al*, 1977). Serology for a range of other viruses was negative.

In a larger study of 12 cases of JDM, 10 (83%) showed elevated titres of complement fixing antibodies to coxsackie B virus compared to 25% of matched controls. No such difference was found with the other viruses studied (Christensen *et al*, 1986).

A few other cases of myositis have been shown to have serological evidence of coxsackie B virus infection (Schiraldi and Iandolo, 1978; Dubowitz, 1976).

In contrast, a study looking for serological evidence other viruses in IIMD, including rubella, measles, influenza A and parainfluenza have been negative (Koch *et al*, 1975). Myositis has been associated with a closely related enterovirus, Echo 9 (Jehn and Fink, 1980) and in association with hypogammaglobulinaemia (see above).

4.5 Virus isolation

Coxsackie B virus was first isolated from muscle in a young child with fatal infection producing a diffuse myositis (Sussman *et al*, 1959). Coxsackie A2 was found in the stool, but not skeletal muscle in a 14 year old with rheumatoid arthritis, polyarteritis and dermatomyositis (Zweymuller, 1953). Virus has also been isolated from cases of chronic myopathy in a few other cases (Tang *et al*, 1975) and by serology and immunofluorescence of muscle (Kuroda *et al*, 1986).

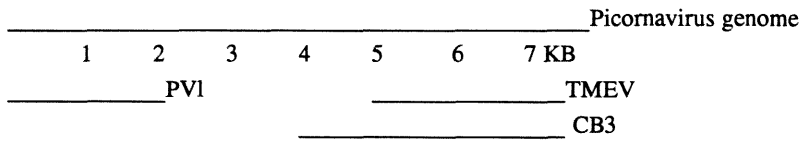
4.6 Detection of viral RNA

The first claim for the detection of viral RNA in muscle from patients with IIMD used cDNA probes to the 3' end (containing the RNA polymerase coding region) of coxsackie B group viruses (Bowles *et al*, 1987). By using slot-blot hybridisation to extracted RNA, with these probes and β-tubulin cDNA, a ratio of binding was obtained, known as the hybridisation index. An index of greater than 2.67 was considered positive. By this calculation 4/7 cases of childhood dermatomyositis and 1/2 cases of adult polymyositis were positive for viral RNA. None of the 10 controls were considered positive, although all had some binding to the coxsackie B virus specific probe. None of the controls were of inflammatory muscle disease.

Two studies to detect virus by *in situ* hybridisation have been published:

(i) The first study (Rosenberg *et al*, 1989) used radio-labelled RNA probes to TMEV, polio 1 (PV1) and coxsackie B3 (CB3), sequenced against the regions of the genome indicated in the diagram below:

Approximate positions and lengths of probes used by Rosenberg *et al* (1989)



Muscle biopsies from 5 cases of adult dermatomyositis, 4 cases of childhood dermatomyositis, 5 cases of polymyositis and 19 controls were examined. 3 patients with adult dermatomyositis were considered positive for TMEV. Signal was seen in similar parts of the section to areas of HLA-DR staining in parallel sections and was therefore thought to be within interstitial macrophages. The other cases were negative with TMEV probes and all cases negative with other probes.

These findings were somewhat difficult to explain as TMEV is not known to be a human pathogen.

(ii) The other *in situ* study used an enterovirus specific cDNA probe homologous to sequences of the coxsackie B3 genome (Yousef *et al*, 1990), closely related to the coxsackie probe used in the previous study. Of the 13 cases of IIMD studied, 6 (46%) were positive with viral RNA present within myocytes. In this study there was no significant difference in clinical or pathological features between positive and negative cases. Also a monoclonal antibody specific for the enterovirus group antigens was negative in all cases. One criticism of the methodology is that RNase controls to confirm that the probe bound RNA within muscle tissue were not performed, and the photomicrographs used to illustrate positive cases were not convincing.

The following recent studies using the highly sensitive and specific polymerase chain reaction were published during the course of my research period:

(i) The first looked for the presence of genomes of a number of candidate viruses (Leff *et al*, 1992). Primers for coxsackie viruses, mumps, encephalomyocarditis virus, adenoviruses, HTLV I and II, and HIV were constructed, with insulin receptor mRNA as a positive control. Using extracted RNA from frozen muscle biopsies from 44 cases of IIMD, none had any demonstrable viral genomes present and all were positive with the control primers.

(ii) A second PCR study looked for the presence of coxsackie virus RNA in 39 cases of IIMD and 19 control cases (Leon-Monzon and Dalakas, 1992). Nucleic acids were extracted from frozen sections in each case and subjected to either DNA amplification for myosin DNA or RT-PCR to demonstrate coxsackie virus RNA. All cases were positive for myosin DNA and negative for virus RNA. No amplification of a host "control" mRNA was carried out to confirm that there was adequate RNA preservation in the extracted RNA samples.

4.7 Murine models

Coxsackie B1 virus inoculated into neonatal CD1 Swiss mice develop a disease with clinical, electromyographic and histopathological features of human PM with muscle fibre necrosis and an inflammatory cell infiltrate. The changes persisted for several weeks after virus could be detected (Strongwater and Schitzer, 1983; Strongwater *et al*, 1984) This myositis predominantly involves proximal muscle groups and is dependant on the strain of mice used (Ray *et al*, 1979).

It has been shown that thymectomised mice recover from the initial infection but do not develop the persistent myopathic change, suggesting that T lymphocytes are required (Ytterberg *et al*, 1988). Also the myositis does not occur in *nu/nu* mice (Ytterberg *et al*, 1987)

Encephalomyocarditis virus is a related picornavirus with myopathic (EMC-221A) and diabetogenic (EMC-D) strains. When injected into BALB/c mice produce a severe myositis with no diabetes (EMC-221A) or a mild myositis with elevated blood glucose (EMC-D). Other strains of mice (NZB x NZW) only get a mild disease (Miller *et al*, 1987).

Confirming the persistence of virus in these models studies using *in situ* hybridisation have been carried out. Radio-labelled RNA probes have demonstrated viral RNA in EMC-221A infected mice within myocytes, both in and away from sites of inflammation. Inflammation in skeletal muscle lasts 3-4 weeks, but no virus cultured after 2 weeks. Virus was seen by *in situ* hybridisation for 3-4 weeks (Cronin *et al*, 1988).

These animal studies describe a proximal inflammatory myopathy in mice, clearly induced by coxsackie B viruses or encephalomyelitis virus, which bears many similarities with human myositis.

4.8 Other evidence

Myocarditis for which there is good evidence of coxsackie viral aetiology (see section 3.3.8) is also associated with IIMD is a significant number of cases (Haupt and Hutchins, 1982; Oka and Raasakka, 1978; Denbow *et al*, 1979), thus providing circumstantial evidence for a coxsackie virus aetiology for IIMD.

The strong association between echovirus and myositis in immune deficient patients has already been discussed (see section 1.9.1). Echovirus has also been linked to muscle damage in a healthy patient (Josseison, 1980)

4.9 Summary

There is a significant amount of evidence for picornaviruses in the aetiology of IIMD, but much of it is circumstantial. Those rare cases, discussed above, where virus has been isolated from muscle, on closer inspection are not typical cases of IIMD. The earlier studies claiming to have detected coxsackie virus RNA (Bowles *et al*, 1987; Yousef *et al*, 1990) are the subject of some technical criticism, especially with the more recent negative findings by PCR (Leff *et al*, 1992; Leon-Monzon and Dalakas, 1992).

5. AIMS OF THIS STUDY

This study sets out to investigate the claims of viral presence within skeletal muscle from cases of IIMD. The presence of virus in cases is obviously of great importance in our understanding of the aetiology and pathogenesis of IIMD, and in view of the controversial evidence so far published, further investigation seems justified.

A number of approaches were considered to investigate the presence of virus within tissue from cases:

(i) Direct culture - this method is unlikely to be positive in the biopsies, some of which are several years old and many are fixed in formalin.

(ii) Electron-microscopy - virus particles can be seen by electron microscopy, but because all picornaviruses have a similar ultrastructural appearance definite identification of virus type is impossible by this technique. There is also the problem of distinguishing virus from glycogen (Katsuragi *et al*, 1981). This technique, used alone, is therefore unlikely to resolve the issue with any certainty. However, the combination of electron microscopy with other techniques may yield important evidence as to the nature of "picornavirus-like particles".

(iv) Immunocytochemistry - detection of virus antigen by antibodies within tissues would be a possibility. The main drawback with immunocytochemistry is the lack of any commercially available antibody to detect coxsackie viruses within tissues. This approach would therefore be time consuming and involve the development of monoclonal or polyclonal antibodies in an animal model.

(v) The polymerase chain reaction - this is a highly sensitive and specific measure of presence of nucleic acids, which has been used previously to detect virus RNA from tissues. Several published sequences for primers directed against the coxsackie viruses are available, and once developed the PCR gives very quick results (a few hours). The main disadvantage of the PCR is the absence of any cellular localisation, so that a positive signal would not distinguish whether the virus RNA was in the fixative, paraffin wax, muscle, blood or connective tissue of the patient. The information provided by PCR is therefore limited.

(vi) In situ hybridisation - in situ hybridisation is specific, sensitive and provides precise cellular location of the virus. This technique has been greatly used in The Department of Pathology at Leicester, with considerable success. Other advantages include the correlation of virus presence with other morphological changes present within muscle from cases e.g. the presence of an inflammatory cell infiltrate and muscle fibre necrosis. Also the presence of virus could be compared with the expression of immunological markers on muscle fibres, e.g. HLA-DR (Zuk and Fletcher, 1988), which would be of great importance to the understanding to the immunopathological process in IIMD as well as other autoimmune diseases where HLA-DR expression has been demonstrated on epithelial cells.

These advantages made *in situ* hybridisation the method of choice for this study. The PCR was also chosen as a more sensitive measure of virus presence and giving additional confirmation of the findings by *in situ* hybridisation.

The main aims of this study are summarised as:

1. Develop probes to the coxsackie B group viruses suitable for *in situ* hybridisation.
2. Assess the prevalence of these infectious agents in muscle tissue obtained from cases of IIMD and compare it to that found in control cases (muscle disease of known cause such as dystrophy and denervation; normal muscle and muscle from other conditions in which an inflammatory cell infiltrate is present such as that involved by tumour or infection).
3. Compare the localisation of the virus with the various morphological changes seen in IIMD.
4. Compare the localisation of the virus with fibre type and expression of HLA-DR by muscle fibres.
5. Correlate presence of virus with clinical features e.g. skin involvement, malignancy, anti-Jo-1 antibody in the serum.
6. Draw some conclusions as to the possible roles played by coxsackie B group viruses in the pathogenesis of IIMD.

In addition to the above, all the cases that have suitable tissue available will have electron microscopy carried out in order to identify the presence of any cases with "picornavirus like" particles, and correlate their presence with the results of molecular biological studies.

PART 2

IN SITU HYBRIDISATION STUDY

1. GENERAL PRINCIPLES

1.1 Introduction

In situ hybridisation depends on the specific annealing of a nucleic acid probe to complimentary RNA or DNA sequences within cells, either within tissue sections or cytological preparations. Detection of a label attached to the probe allows demonstration of specific nucleic acid sequences within cells.

The technique has been used since 1969 (John *et al*, 1969; Buogiorno-Nardelli and Amaldi, 1969; Gall and Pardue, 1969). Most early studies have used radiolabelled probes to detect DNA, however, recently attention has turned to detection of mRNA with increasing use of non-radioactive labels.

1.2 Specimen preparation

Optimal fixation is important for good *in situ* hybridisation results. Fixation allows preservation of cell morphology and nucleic acids, particularly for RNA detection, which is very susceptible to digestion by endogenous ribonucleases. In order to prevent this tissues should either be fixed or frozen as soon as possible after surgical excision. To prevent contamination from exogenous nucleases sterile equipment and gloves should be used in further handling if tissues.

Many different fixatives can be used, however cross-linking fixatives, such as formalin and paraformaldehyde, give the best retention of nucleic acids, by forming chemical bonds with cellular proteins (Singer *et al*, 1986).

It is important to coat the glass slides to ensure section adhesion, either poly-L-lysine (Huang *et al*, 1983) or aminopropyl-triethoxysilane (Van Prooijen-Kneght *et al*, 1983) are suitable.

1.3 Pretreatments

A number of pretreatments are used, both to reduce non-specific background and increase probe penetration.

Pretreatments may depend on the method of fixation, for example 0.1M hydrochloric acid reduces non-specific background in formol-sublimate fixed tissue.

Acetylation can also used to reduce binding to basic groups within the tissue (Hayashi *et al*, 1978).

Permeabilisation of the section is necessary for formalin fixed tissue to reduce the effect of cross-linking. This can be achieved by proteolytic digestion with proteinase K (Pringle *et al*, 1987) or pronase (Brigati *et al*, 1983).

A prehybridisation solution applied prior to addition of the probe contains high concentrations of single stranded sheared DNA which act as a blocker of sites within tissue binding nucleic acid non-specifically.

1.4 Probes

Several different types of probes may be used:

(i) Double-stranded DNA probes - these have to be denatured before use and are usually labelled by random priming or nick translation.
(ii) Single-stranded DNA probes - these are usually cloned in the bacteriophage vector M13 and do not need to be denatured.
(iii) Synthetic oligonucleotides - single stranded DNA probes can be synthesised using phosphoramadite chemistry (Beaucage and Caruthers, 1981) on a DNA synthesiser. Labelling is usually at the 3' end with terminal deoxynucleotidyl transferase or at the 5' end with polynucleotide kinase or chemically via an amino-link (Chu and Orgel, 1985).
(iv) Single-stranded RNA probes (riboprobes) - these are generated from cDNA in specially constructed RNA expression vectors.

The various advantages and disadvantages of the different probes are summarised below:

Advantages	*Disadvantages*
ssRNA	
RNA:RNA hybrids are very stable	Sub-cloning required
No probe denaturation	Avoid RNase contamination
Probes strand specific	Narrow optimum hybridisation
Free of vector sequence	temperature
Template removal easy	
Post-hybridisation RNase treatment removes unbound probe	
dsDNA	
No sub-cloning required	Probe denaturation required
Wide choice of labelling	Re-annealing occurs during
Hybridisation temperature less critical	hybridisation
Amplification of signal by networking with vector sequence	Hybrids not very stable
	Gel purification to remove vector sequence
ssDNA	
Free of vector sequence	Sub-cloning to vector required
No probe denaturation	Hybrids not very stable
No re-annealing during hybridisation	Technically difficult
Synthetic oligonucleotides	
No cloning	Limited labelling methods
No self-hybridisation	Small size limits amount of
Good target penetration	label
Can be constructed from sequence deduced from amino acid data	Subject to "design" errors
	Need DNA synthesiser
Can avoid homology with related sequences	Hybrids less stable than RNA probes
Stable	Need sequence data
Low background	Errors in published sequence
Simple protocol	
No need for cDNA	
Probes can be designed to look for alternatively spliced mRNA	

1.5 Labelling

Originally radioactive molecules were used for labelling (Gall and Pardue, 1969) and these are still widely used. They have the advantage of being readily incorporated into probes and very sensitive, although they do not all give precise cellular resolution and take several days exposure to produce results. Non-radioactive probes give clear sub-cellular location and quick results but may not be as sensitive. Some of the labelling methods commonly used are compared below:

Table 5 - Comparison of probe labelling/detection methods

Label	Resolution	Sensitivity	Exposure (days)	Stability (weeks)
^{32}P	+	++	7	0.5
^{35}S	++	+++	10	6
^{3}H	+++	+++	14	>30
Biotin	+++	++	0.16	>52
Digoxigenin	+++	+++	0.16	>52

Two recent studies have found that digoxigenin is as sensitive ^{35}S and more sensitive than biotin (Morris *et al*, 1990; Furunta *et al*, 1990), making it the label of choice in many centres.

Homopolymer tailing method

The homopolymer tailing method is a useful way of adding labelled nucleotides to oligonucleotide probes. Labelling at the 3'-hydroxyl terminus is carried out with an unusual DNA polymerase, terminal deoxynucleotidyl transferase, found in prelymphocytes (Chang and Bollum, 1986). In the presence of a divalent cation (Mg^{++} is preferred for purines and Co^{++} for pyrimidines), nucleotides can be added to the 3' hydroxyl termini of DNA, which must be at least 3 bases in length (Bollum, 1974).

1.6 Hybridisation conditions

A number of variables in the conditions used for hybridisation affect the degree of mis-matching allowed between target and probe, termed "stringency".

(i) Temperature and ionic strength - the optimum temperature for efficient hybridisation of homologous nucleotide sequences is 20-30^{o}C below the melt temperature (Tm) of the specific hybrids. The Tm is the temperature at which 50% of the duplexes are dissociated and is proportional to the G-C content, duplex length in base pairs (L), concentration of monovalent cations (M), and % inter-strand homology (H) (Lathe, 1985). The Tm can be deduced from the equation:

$$Tm\ (^{o}C) = 16.6 \log M + 0.41(\%G+C) + 81.5 - 820/L - 1.2(100-H)$$

In general the inclusion of formamide, a helix destabiliser, into the hybridisation mixture reduces the Tm by 0.7^{o}C for each 1% formamide. However, formamide destabilises A-T rich hybrids disproportionately more than G-C rich hybrids. Also the above equation refers to DNA-DNA hybrids, not RNA-DNA and RNA-RNA hybrids which are more stable and therefore have higher melt temperatures.

Another important factor is that G-T or G-A mismatches are less destabilising than A-A, T-T, C-T or C-A mismatches (Ikuta *et al*, 1987) and mismatches in the middle of an oligonucleotide are much more deleterious than mismatches at the ends.

(ii) Accelerators - inclusion of dextran sulphate increases effective probe concentration and hybridisation rates by volume exclusion. Dextran sulphate has the added advantage of reducing non-specific binding of probe to positively charged molecules in the tissue.

(iii) Buffers - Tris/phosphate/sodium citrate based buffers are used to maintain neutral pH.

(iv) Probe concentration - low probe concentrations are important for the reduction of non-specific binding.

(V) Blocking agents - a number of other macromolecules can be included in the hybridisation mixture to reduce background, including salmon sperm DNA, transfer RNA and Denhart's solution.

1.7 Post-Hybridisation washes

Mismatched and non-specifically bound probe can be washed off in a salt solution, and by varying the temperature, formamide and ionic concentration of this solution, the specificity of the final detected signal can be altered. Unbound RNA probes can be removed with RNase A and likewise DNA probes with Sl nucleases.

1.8 Controls

In order to confirm the specificity of signal a number of controls are usually employed:
(i) Predigestion of the section with RNase or DNase will confirm the targets as nucleic acids.
(ii) Negative control probes (designed to unrelated RNA species) will ensure binding is due to specific hybridisation.
(iii) Omission of probe from the hybridisation mixture, to exclude non-specific reactions in the detection systems.

To demonstrate RNA/DNA preservation in the tissue a positive control probe should also be included. This will exclude one cause of false negative results.

2. ISH PROBES FOR THIS STUDY

In view of the time available and previous experience within the Department of Pathology at Leicester, I decided to use synthetic oligonucleotide probes, labelled with non-radioactive haptens, for *in situ* hybridisation. These have many advantages, listed previously, and the Department has had considerable success with their use, producing high resolution signal without the need of excessive permeabilisation of the tissue, due to the small size of oligonucleotides. The coxsackie virus genome being it's own mRNA provides a simple target to design probes to and 3 out of the 6 coxsackie B group viruses having been fully sequenced at the time of study (see below).

Oligonucleotides of 30 bases in length were synthesised. Shorter sequences would form less stable hybrids and react with a broader range of viruses including ones of no interest to this study such as polio and rhinovirues, whereas longer sequences would not be homologous to all three coxsackie B virus types. Also probes of this length allow more flexible limits of stringency to be used and still remain specific as well as being long enough to diminish the chance of cross-hybridisation with similar sequences in the human genome.

2.1 Coxsackie B group virus oligonucleotides

The initial aim was to detect viral RNA from muscle tissue using *in situ* hybridisation and therefore oligonucleotide probes were designed complimentary to conserved sequences of the genome.

The sequences of 3 out of the 6 coxsackie B virus subtypes is known (coxsackie B3 - Klump *et al*, 1990; coxsackie B1 - Izuka *et al*, 1987; coxsackie B4 - Jenkins *et al*, 1987). By comparing these genomes regions of homology were identified. Regions of high homology tended to occur in particular regions: the 5' non-coding terminus (nucleotides 1-741 from Klump *et al*, 1990) sequences coding for the polypeptide 2C (nucleotides 4041-5026) and the 3' non-coding region (nucleotides 7296-7399 from Klump *et al*, 1990). By choosing these regions of high homology it is hoped that as many different strains of coxsackie virus as possible will be detected, including mutants, who presumably need to conserve these regions as well.

Suitable sequences were chosen, and these were selected so that they did not occur too closely to each other (greater than 20 bases apart) to avoid any possibility of

interference of probe hybridisation once they have been labelled by the addition of a tail. It was not possible to design 6 oligonucleotides with perfect homology, so in some cases one of 2 bases was inserted in specific positions (done simply by opening the ports to both nucleotides at this position during synthesis).

One sequence (B/ECHO) was chosen which had a low GC content. This was selected because, although the GC content was much lower than optimum, it represents a sequence that is deleted from enteroviruses other than coxsackie B group and Echo viruses, and therefore may offer a more specific diagnosis.

Details of the selected sequences together with the number of predicted mismatches between the various sequenced virus strains is given below.

Table 6 - Coxsackie B virus oligonucleotides for ISH

Probe	Sequence (5'→3' direction)	Nucleotide position*	GC content	Maximum number of mismatches		
				B1/3/4*	A9**	A21**
CXB1	AAC.AGG.CGC.ACA.AAG.GTA.CCG.T/GA/GA.TAC.CAG	86-57	50-57%	2	2	6
CXB2	GCA.GGC.CGC.CAA.CGC.AGC.CAC.CGC.CAC.GGT	376-347	76%	0	3	5
CXB3	TCT.TCG.CAC.CAT.GTC.TGT.ATC/T.AGA.GCG.TCC	424-395	50-53%	2	4	11
CXB4	GTG.CTC.CGC.AGT.TAG.GAT.TAG.CCG.CAT.TCA	488-459	53%	0	1	3
CXB5	TCG.GTT.CCG.CTG.CAG.AGT.TGC.CCG.TTA.CGA	543-514	60%	0	0	8
CXB6	GGA.TGA.ACT.CCT.CAT.ATT.CGT.GCT.CC/TC.CGT	7214-7185	50-53%	2	3	15
B/ECHO	AGC.CAA.TTT.AAA.TTG/A.TTT.CAA.ATT.GC/AC.TCT	7332-7303	23-30%	4	4	deleted

* Klump *et al*, 1990

** Chang *et al*, 1989

***Hughes *et al*, 1989

2.2 Control probes

In order to demonstrate RNA preservation in the tissues examined it is necessary to use oligonucleotides complimentary to a normally expressed host mRNA. A number of possibilities were considered including a range of muscle structural proteins: actin (Taylor *et al*, 1988), myosin (Seidel and Arnold, 1989) and desmin (Zhenlin *et al*, 1989). Although all are expressed in muscle most of these appear to only be highly expressed during myogenesis, so would not be present in high enough amounts to detect in mature skeletal muscle by ISH. The more highly expressed mRNA species tend to code for relatively short lived proteins such as enzymes (Carter and Malter, 1991). Of those expressed in muscle a number of mRNA species have been sequenced including glycogen synthetase (Browner *et al*, 1989), creatine kinase (Trask *et al*, 1988) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Tso *et al*, 1985). Of these creatine kinase mRNA and G3PDH mRNA expression appeared high enough to detect by ISH (as judged by the published Northern blots). G3PDH was chosen because of it's high expression (3.6% of muscle mRNA - Piechaczyk *et al*, 1984), but because it is a key enzyme in glycolysis, expression is higher in type II fibres and therefore may offer the further advantage of being able to differentiate fibre types.

3 sequences complimentary to G3PDH mRNA were chosen of 29-30 bases in length and with a GC content of 50-60% to ensure stable hybrids. The sequences were spaced out to avoid any possible interactions during hybridisation, and regions conserved between rat and human mRNA were chosen so that they would be more likely to work in animal tissues. Sequences are given below:

Table 7 - G3PDH oligonucleotides for ISH

Probe	Sequence (5'→3' direction)	Nucleotide position*	GC%
G1	AGC.CCA.GCC.TTC.TCC.ATG.GTG.GTG.AAG.AC	326-297	57
G2	GGC.AGT.GAT.GGC.ATG.GAC.TGT.GGT.CAT.GAG	545-515	53
G3	CAT.GAG.GTC.CAC.CAC.CCT.GTT.GCT.GTA.GCC	982-952	60

* sequences from Tso *et al*, 1985

Other oligonucleotides already available in the department include oligo d(T) (Pringle *et al*, 1989) which detects the polyadenylated tails of mRNA molecules and oligonucleotides complimentary to mitochondrial rRNA (Chester *et al*, 1990). The oligo d(T) probes have the theoretical disadvantage of detecting the polyadenylated tail and the mitochondrial probes detect a ribosomal RNA molecule, therefore neither may be representative of true mRNA preservation within a cell. These different oligonucleotides were tested on human skeletal muscle to assess their usefulness.

2.3 Oligonucleotide synthesis

Oligonucleotides are synthesised on an oligonucleotide synthesiser using phosphoramadite chemistry (Beaucage and Caruthers, 1981). Briefly a sequential chain of activated monomers are added to an insoluble support. The 3' phosphate group of the activated monomer is added to the 5' hydroxy group on the growing chain, forming a phosphite triester intermediate. This is then oxidised to a phosphotriester. Coupling must be carried out in anhydrous conditions as phosphoramadites react with water. The new monomer has it's 5' hydroxy group blocked by a dimethoxytrityl protecting group, which is removed by a deproteation step (with dichloroacetic acid) before further bases are added. At the end the DNA can be released from the supporting resin by ammonium hydroxide and other protecting groups on the bases are removed.

All the oligonucleotides used in this study were synthesised at the Department of Biochemistry, University of Leicester.

2.4 Probe purification and labelling

Once the oligonucleotides have been synthesised they are purified further to remove unincorporated nucleotides, prematurely terminated oligonucleotides and other chemical impurities that may hinder further enzyme activity.

In order to separate the nucleic acids they are precipitated in cold absolute ethanol with 0.1 M NaCl, followed by vacuum drying (see appendix 2). The concentration of DNA is calculated by measuring the optical density (OD) in UV light at 260 nm in lead crystal cuvettes after first zeroing the meter with 1 X TE.

The concentration of nucleic acid depends on what type it is, a reading of 1 is equivalent to: 30 μg/ml for ssDNA, 40 μg/ml for RNA and 50 μg/ml for dsDNA.

e.g. an OD of 0.26 for oligonucleotide diluted 1:500 is equivalent to 0.26 X 30 X 500 μg/ml or 3.9 μg/μl.

The labelling is carried out using the homopolymer tailing method to add nucleotides to the 3' hydroxy terminus with terminal deoxynucleotidyl transferase in the presence of a divalent cation (see appendix 2). Initially a cytosine "spacer" nucleotide was used to help separate the digoxigenin-11-dUTP bases to avoid the theoretical possibility of steric hindrance when the antibody is bound during detection.

Another labelling reaction was carried (see appendix 2) out for comparison which involved incorporating alyll amine dUTP molecules by the homopolymer tailing method described above followed by purification then chemically linking digoxigenin-NHS (digoxigenin-3-o-methycarbonyl E amidocaproic acid hydroxy-succinamide ester) to the alyll amine groups and to a 2' amino group added to the 5' end of the oligonucleotides during synthesis. This chemical reaction is extremely efficient and some workers in the laboratory have found it better than conventional homopolymer labelling. However, I found only a marginal improvement in labelling and as the procedure is more time consuming and expensive I did not continue with it.

Once the labelling reaction has been incubated for 2 1/2 hours at 37°C the labelled oligonucleotides are purified by passage through a column of sephadex G50 (Pharmacia 17-0045-01) pre-swollen in 1 X TE, pH 8.0 (see appendix 2). The oligonucleotide is added to the top of the column which is placed over a clean eppendorf tube and centrifuged. Only full length probe is produced from the column, all the smaller molecules, including unincorporated nucleotides, remain trapped within the sephadex beads.

2.5 Test strips (see Appendix 2)

In order to ensure adequate incorporation of the digoxigenin-11-dUTP, serial dilutions of the labelled oligonucleotide are made by adding a diluent (containing salt and SSDNA). These dilutions are spotted on to a nitrocellulose filter in 1 μl amounts and baked on at 80°C. After a blocking step with bovine serum albumin to

prevent non-specific attachment of antibody to the nitrocellulose, the labelled oligonucleotide detected with anti-digoxigenin antibody which has been conjugated with alkaline phosphatase (Boehringer Mannheim). Unbound antibody is washed off and the remaining antibody can then be visualised by dipping the filter into a substrate buffer of nitroblue tetrazolium (NBT) salt and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). This is developed in the dark for 15 minutes- 12 hours (depending on the level of background). A good labelling reaction will allow visualisation of probe to 10 or 1 pg/µl. An example of a test strip is given below.

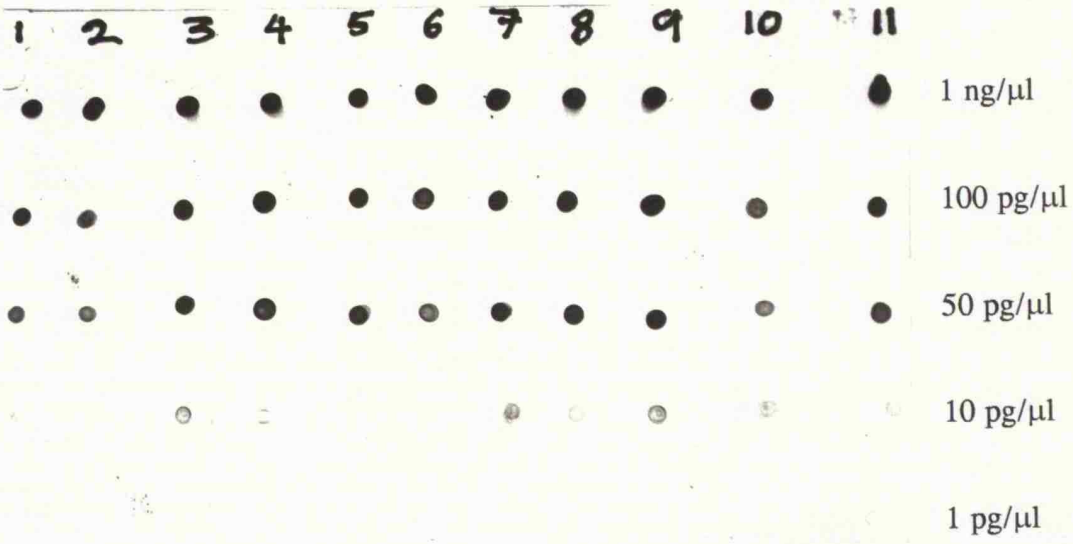


Figure 3 - An example of a test strip showing the results of TdT labelling reactions with digoxigenin-dUTP with 12 different oligonucleotides at different dilutions

3. ISH ON SUCKLING MICE

In order to demonstrate that the oligonucleotides designed against the coxsackie B virus published sequences detected strains of virus infecting animal cells, either cell lines or an animal model was required. Although most strains of coxsackie viruses can be cultured in cell lines (see above) the technique was intended for use in human tissue sections, so it would be more relevant to use mice infected with different strains of virus. Such a model allows the development of an ISH technique directly applicable to human tissue. The probes can be tested both as a "cocktail" or individually against the different strains of virus.

In view of the limited time available I decided that I would not be able to carry this part of the study at Leicester as it would also involve obtaining a Home Office licence. The mice were kindly prepared by Dr Sharon Chambers and Carol Day at the Coxsackie Reference Laboratory, West Park Hospital, Epsom. Details of this are provided below.

3.1 Virus production

Coxsackie B2, B3, B4 and B5; viruses were propagated in confluent monolayers of Vero cells maintained on serum-free medium 199 (Gibco) buffered with 5% Molar N-Tris (hydrocymethyl) methylglycine. Coxsackie A5, A7, A8, A10, Al6 and A21 virus was grown in confluent monolayers of HEL cells maintained as for Vero cells.

Cultures were harvested when showing 80-90% cytopathic effect, freeze-thawed twice and clarified by centrifugation at 1000g for 10 minutes. Virus preparations were stored at -20^{o}C.

All virus strains used were reference laboratory strains.

3.2 Suckling mouse preparation

0.05ml of the virus preparation was inoculated subcutaneously into each of a litter of 8-10 SPF suckling mice. The mice were less than 24 hours old. Litters were examined daily for spastic paralysis (coxsackie B virus strains) or flaccid paralysis (coxsackie A virus strains). One litter remained uninoculated as a control.

All positive mice were killed when they exhibited symptoms. Six mice per antigen were placed in formal saline at +4°C Neutralisation tests were performed on remaining mice to confirm viral identity. Control mice were killed and eviscerated as for test mice.

Blocks of mice were routinely processed and embedded in paraffin wax.

3.3 ISH method

Details of the method are given in appendix 2, briefly:

4μm sections mounted on aminopropyl silane coated slides were dewaxed in xylene and graded alcohols, then washed in hot SSC to denature any RNA secondary structure. They were then treated with proteinase K to expose the target sequences and briefly refixed in 0.4% paraformaldehyde. Hybridisation was overnight at 37°C with 50 μl of buffer containing 0.1ng/μl probe, 30% formamide, 600mM NaCl, 0.1 M phosphate buffer, 10% dextran sulphate and 150μg/ml sheared SSDNA. Post-hybridisation washes consisted of 2 x SSC/30% formamide at 37°C to wash off any mismatched or unhybridised probe. Sections were blocked for 10 mins in 3% Bovine serum albumin, to prevent non-specific antibody binding, followed by incubation with anti-digoxigenin antibody conjugated to alkaline phosphatase at 1:600 (Boehringer-Manheim) for 30 mins. The colour reaction was performed with NBT/BCIP solution in the dark for 5 hours. All slides were counterstained with haematoxylin and mounted in Apathy's medium.

n.b. for oligonucleotides with a low GC content no formamide is used at any stage.

3.4 Results

3.4.1 Nuclear signal

Initial results showed signal in all nuclei as well as within the cytoplasm of degenerate cells (see Figure 4). This nuclear signal was seen with all the coxsackie and G3PDH oligonucleotides. The nuclear signal was reduced but not eliminated by RNase A pretreatment of the tissue (after the proteinase K step), unlike the cytoplasmic signal which was completely removed with RNase. Increasing formamide concentration to 50% eliminated the nuclear signal but not the cytoplasmic signal and reducing probe concentration reduced both nuclear and

cytoplasmic signal equally with all signal disappearing at 0.01-0.002 ng/μl of oligonucleotide in the hybridisation solution. Increasing the concentration of ssDNA in the hybridisation and pre-hybridisation solutions to 500 μg/ml and "capping" the amino link attached to the 5' end of the oligonucleotide with FITC did not affect nuclear or cytoplasmic signal. The cytoplasmic signal showed the expected pattern of distribution (i.e. predominantly in degenerate or necrotic cells with the coxsackie probes and in all skeletal muscle fibres with G3PDH probes. The nuclear signal was, however, unexpected and although showing some features of hybridisation, it clearly was not specific. One possible explanation for these finding is that the nucleotides in the tail were hybridising in a non-specific manner to the nuclei. As the nuclear signal persisted at relatively high formamide concentrations it could not be due to the digoxigenin-11-dUTP nucleotides which would have a much lower melt temperature than cytosine. The cytosine "spacer" was therefore changed to adenine in the labelling reaction. This step completely eliminated nuclear signal, leaving the specific cytoplasmic signal (see Figure 5). The non-specific binding was presumably due to binding to G rich regions within the nucleus by chains of cytosine in the tail of the probe.

3.4.2 Keratin binding

Another artefact noted was the presence of signal in the keratin layer of all skin (see Figure 6), this artefact had previously been noted by others and was seen with all probes used, even if they were labelled with biotin (although less prominent when probes were labelled with biotin than digoxigenin). Signal in the keratin was not affected by RNase A pretreatment or increasing formamide concentration and persisted even at very low probe concentrations. Keratin is rich in basic amino acids which may be reacting with acidic groups of the nucleic acid e.g. PO_3^-. Such binding should be abolished by using an acetylation step which blocks many basic groups in the tissue sections e.g. NH_4^+, thus preventing associations due to charge. Initially I tried the published method for acetylation with 0.25-1% acetic anhydride added to sections in an aqueous (0.1M triethanolamine in DEPC water, pH 8.0) buffer for 10 minutes prior to prehybridisation (Hayashi *et al*, 1978). This method made no difference and on further investigation I discovered that acetic anhydride has a very short half life in the presence of water (only a few seconds), so the procedure was repeated by dipping the slides into acetic anhydride in xylene for 5 minutes immediately prior to hybridisation (see appendix 2). This completely removed keratin staining (see Figure 7), but did not appear to affect hybridisation (which is mediated via hydrogen bonding).

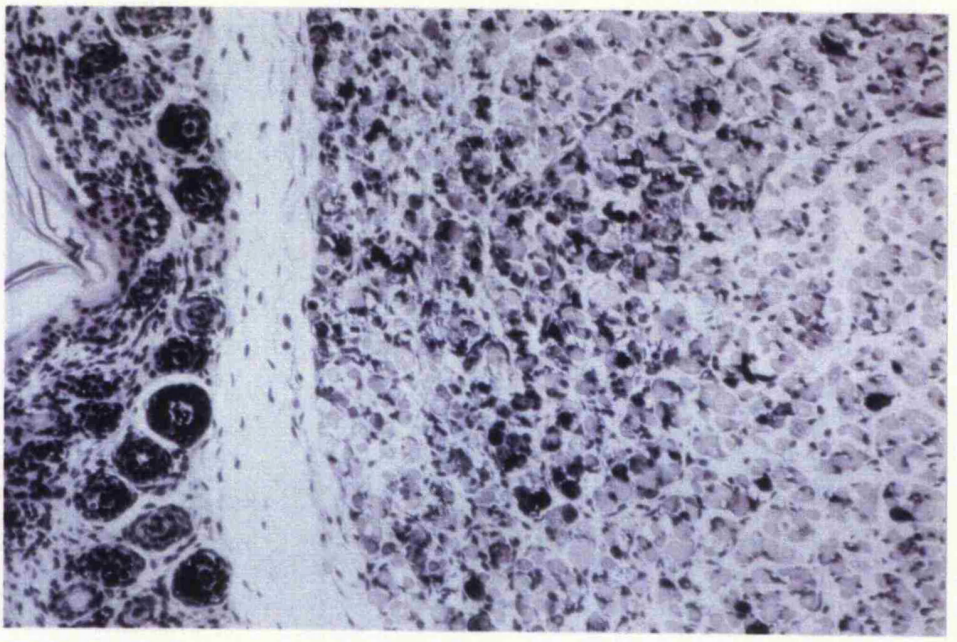


Figure 4 - ISH results using coxsackie B virus probes, labelled with a cytosine "spacer", on mouse section demonstrating nuclear and cytoplasmic signal. Skin and underlying skeletal muscle. Magnification X 130.

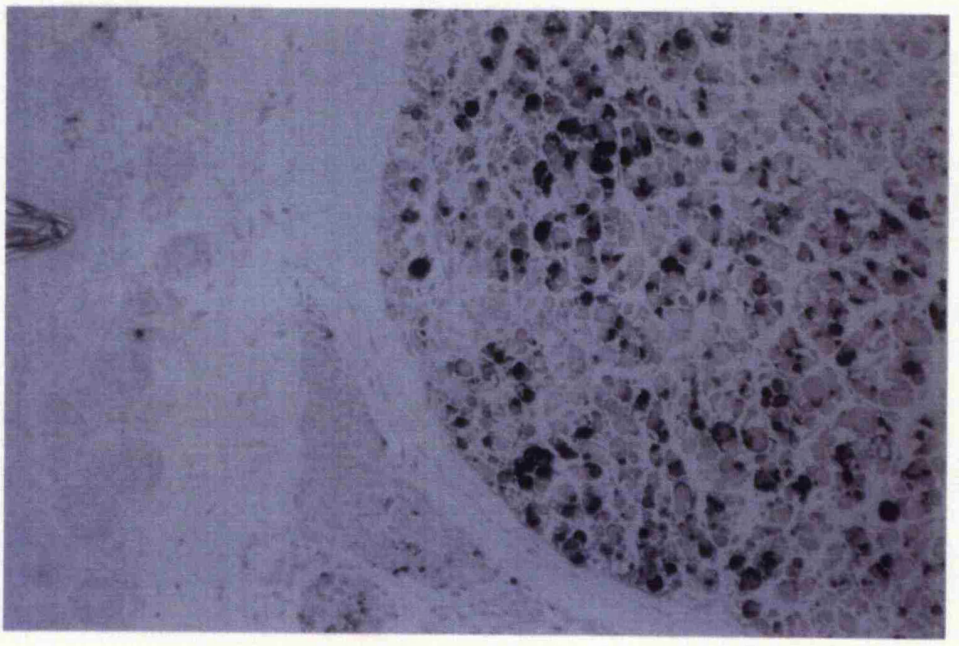


Figure 5 - ISH results using coxsackie B virus probes, labelled with an adenine "spacer", on mouse section. Skin and skeletal muscle. Mag. X 130.

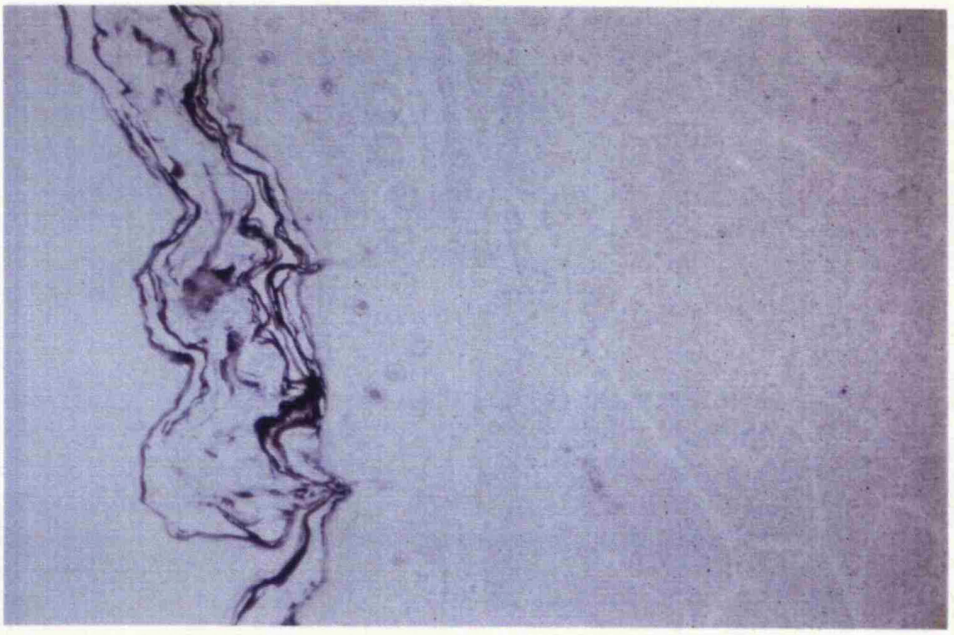


Figure 6 - ISH on mouse section with coxsackie B virus probes showing the keratin binding seen remaining after RNase pretreatment. Skin and underlying brown fat. Magnification X 130.

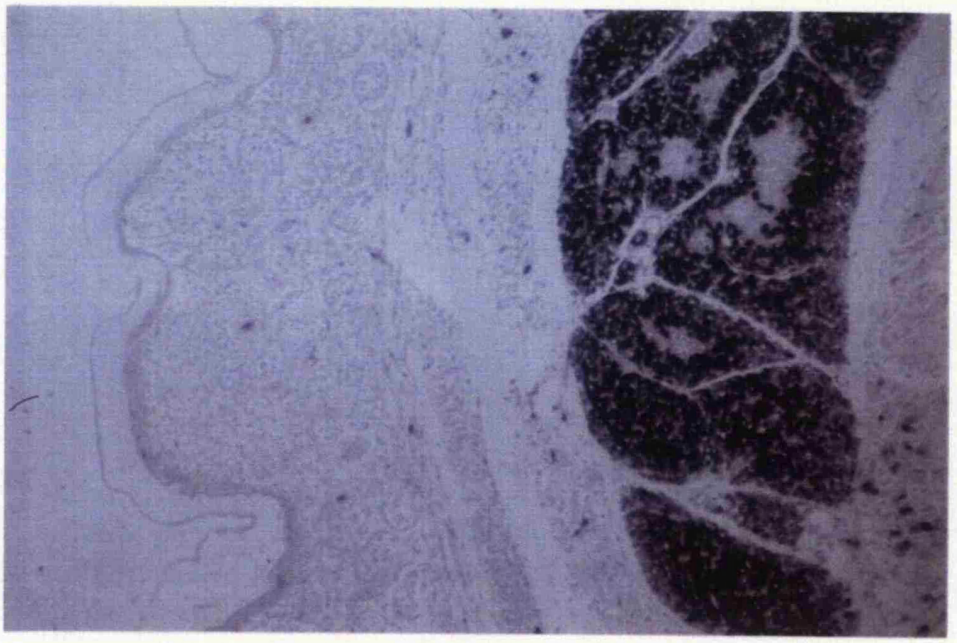


Figure 7 - ISH on murine tissue with coxsackie B virus probes after acetylation of sections with 0.25% acetic anhydride in xylene, showing loss of keratin binding but preservation of hybridisation signal. Skin and brown fat. Mag. X 130.

3.4.3 Signal in connective tissue and cartilage

A small amount of signal was sometimes seen in the connective tissue, this has been described previously as being due to the anti-digoxigenin antibody binding too hydrophobic sites, and can be blocked by a commercial blocking agent (Bland *et al*, 1991).

3.4.4 Distribution of virus in mice

Using a cocktail of oligonucleotides CXB 1-6, virus RNA was detected in all mice infected with coxsackie viruses, but not in sham infected mice. Signal was abolished with RNase A pretreatment and none was seen with similarly constructed and labelled oligonucleotides to EBV RNA.

Two broad patterns of infection were observed:

1. Coxsackie B1, B2, B3, B4 and B5.

All coxsackie B strains examined showed a focal distribution in skeletal muscle involving the front limb girdle, head and neck with mild myocardial involvement. Involvement of the salivary glands, brown fat, exocrine pancreas, thyroid gland, liver and hair roots was also noted. Signal was also detected in the central nervous system (CNS) within anterior horn cells of the cervical and thoracic spinal cord, brain stem nuclei and occasional cells in the cerebral cortex. In occasional sections virus was seen in the walls of major vessels leaving the heart, peripheral nerves and occasional cells at the edge of lymph nodes and the thymus. (See Figures 8-15)

2. Coxsackie A5, A7, A8, A10, A16 and A21.

All produced a much more diffuse skeletal muscle infection with involvement of both limb girdles, the diaphragm and tongue. Signal was also seen in the myocardium and focally within brown fat. No involvement of the CNS was observed except for A16 which showed involvement of only very occasional neurones in the same regions of the CNS as coxsackie B strains. (See Figures 16-19)

No signal was seen in other tissues including the lung, kidney, yellow fat or bone marrow with any of the strains of virus.

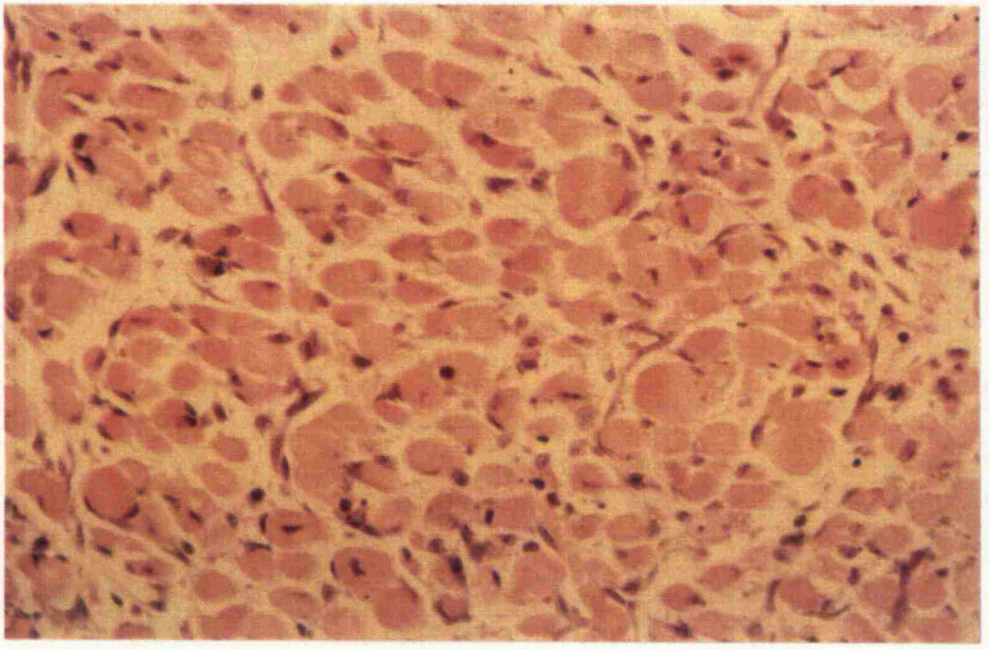


Figure 8 - Haematoxylin and eosin stain showing degenerate and necrotic skeletal muscle fibres in mouse infected with coxsackie B3 virus. Magnification X 500.

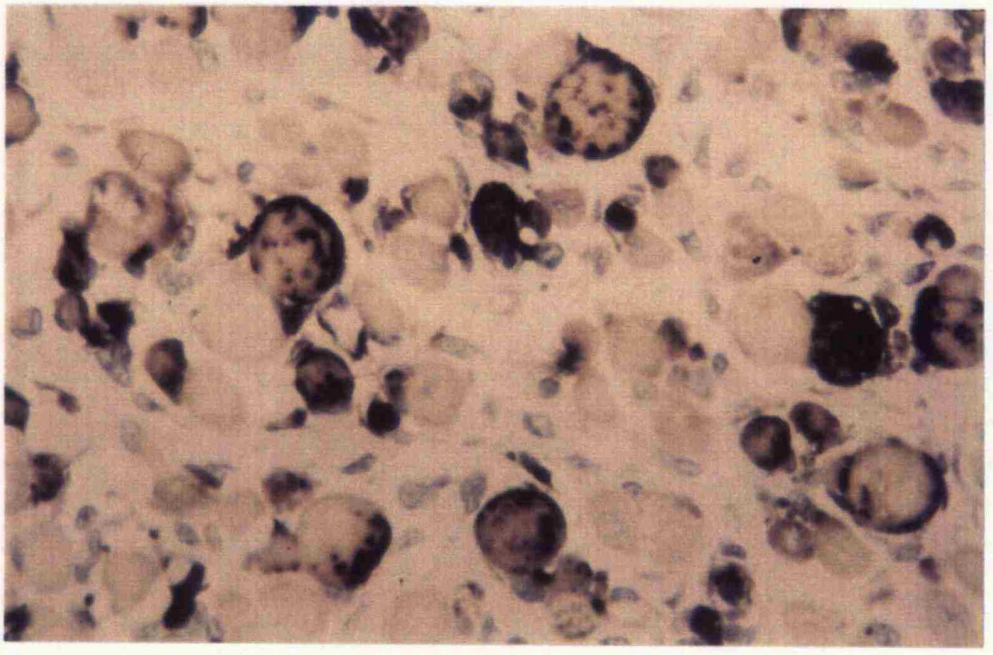


Figure 9 - *In situ* hybridisation with cocktail of coxsackie virus oligonucleotides showing focal distribution of virus RNA in skeletal muscle. Magnification X 810.

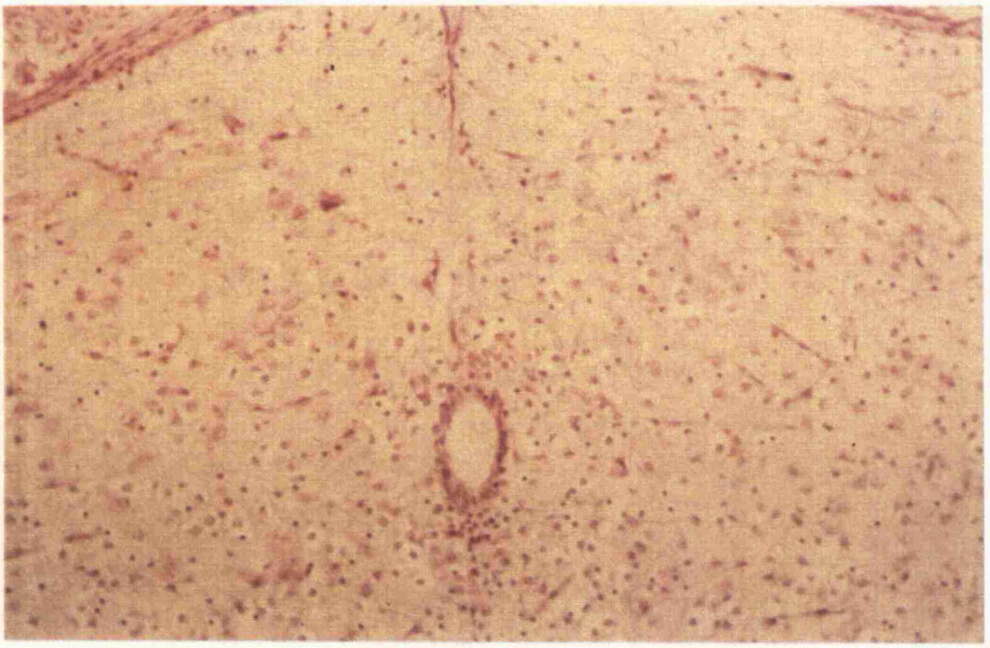


Figure 10 - Haematoxylin and eosin stained section showing thoracic spinal cord from mouse infected with coxsackie B3 virus. Magnification X 210.

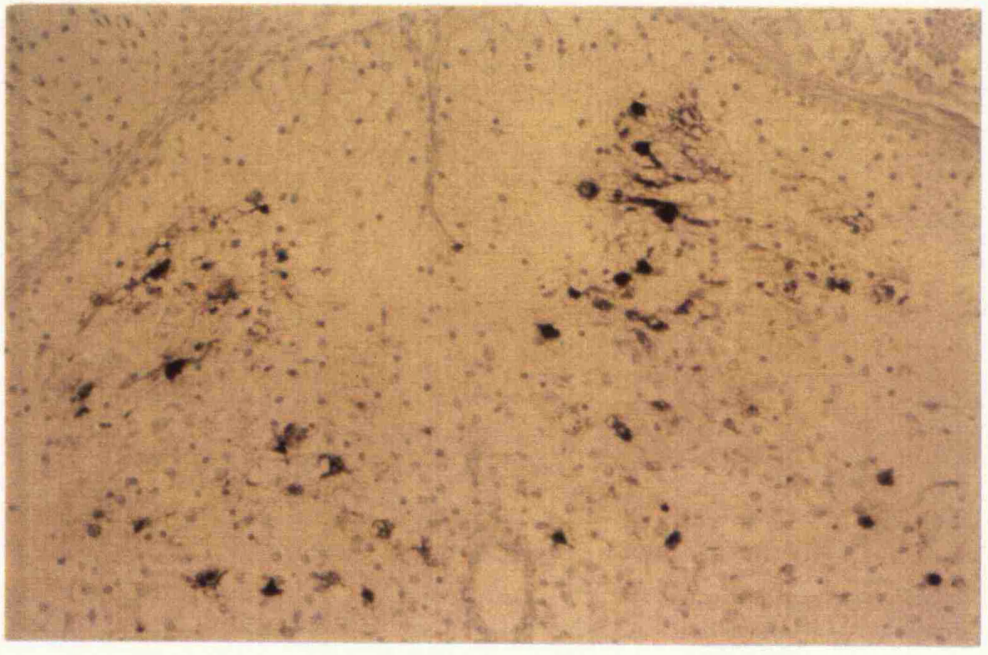


Figure 11 - *In situ* hybridisation with coxsackie virus oligonucleotides showing the presence of virus RNA in the anterior horn regions of the spinal cord. Mag. X 210.

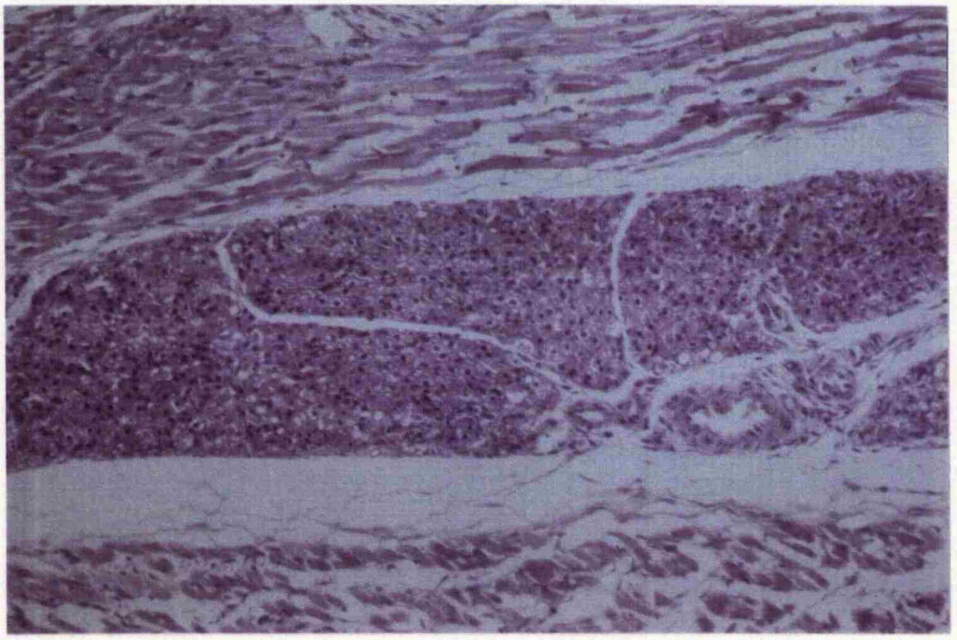


Figure 12 - Haematoxylin and eosin stained section showing brown fat lobules from mouse infected with coxsackie B3 virus. Magnification X 210.

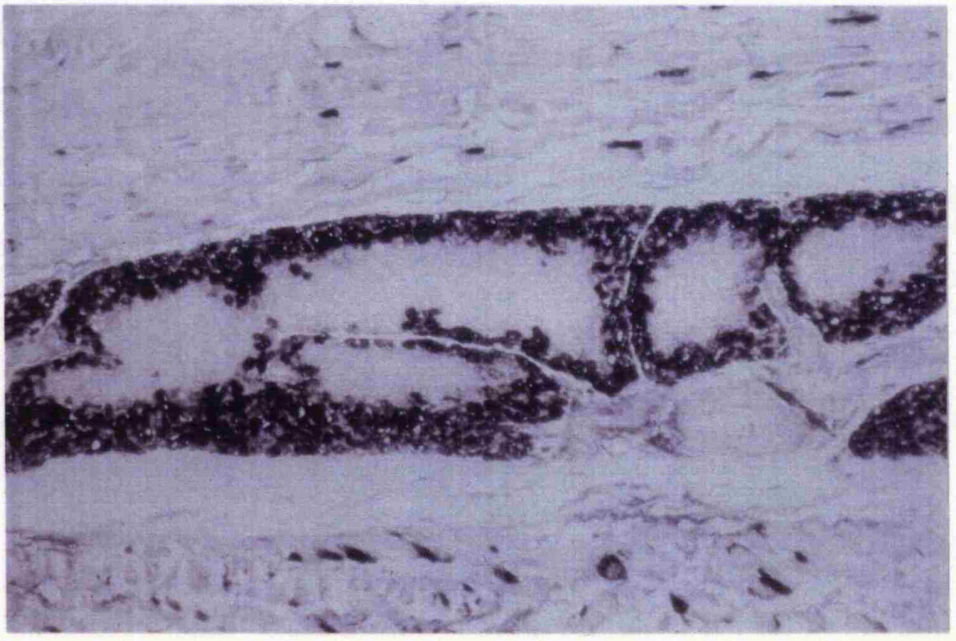


Figure 13 - *In situ* hybridisation with coxsackie virus oligonucleotides showing the presence of virus RNA distributed around the periphery of brown fat lobules. Magnification X 210.

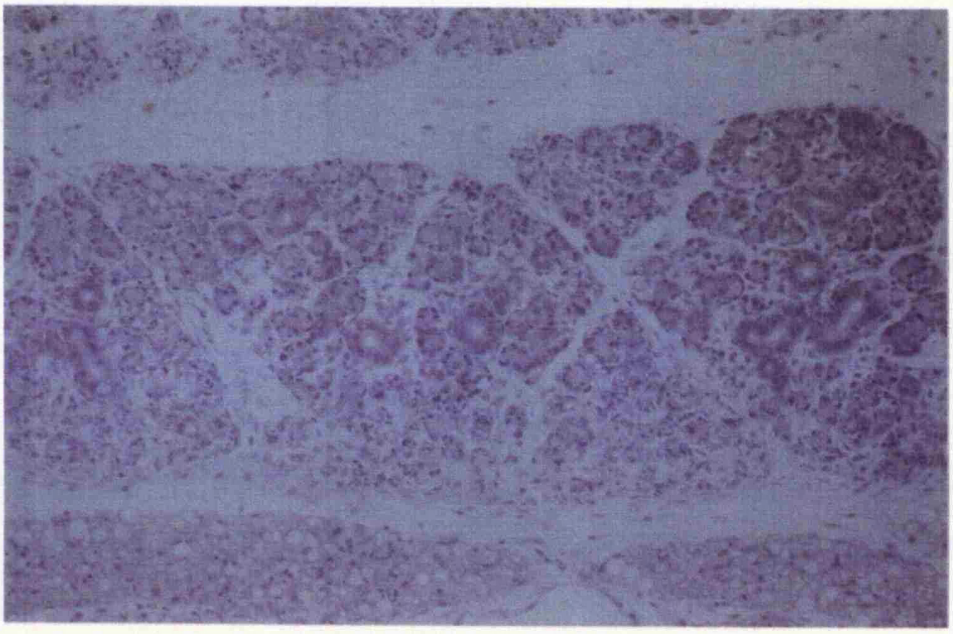


Figure 14 - Haematoxylin and eosin stained section showing salivary gland from mouse infected with coxsackie B3 virus. Magnification X 130.

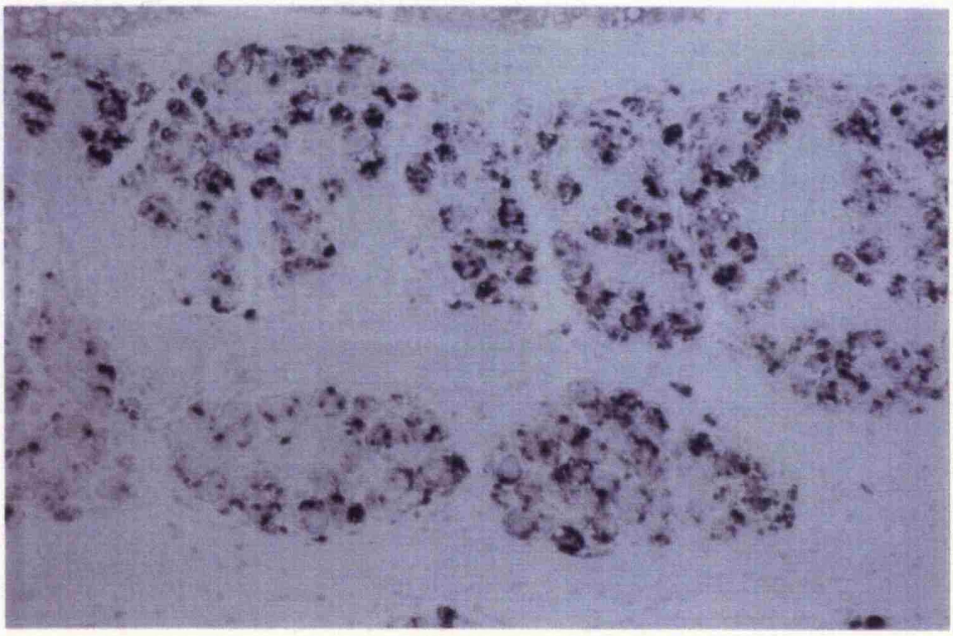


Figure 15 - *In situ* hybridisation with coxsackie virus oligonucleotides showing the presence of virus RNA in the acinar epithelium of salivary gland. Mag. X 130.

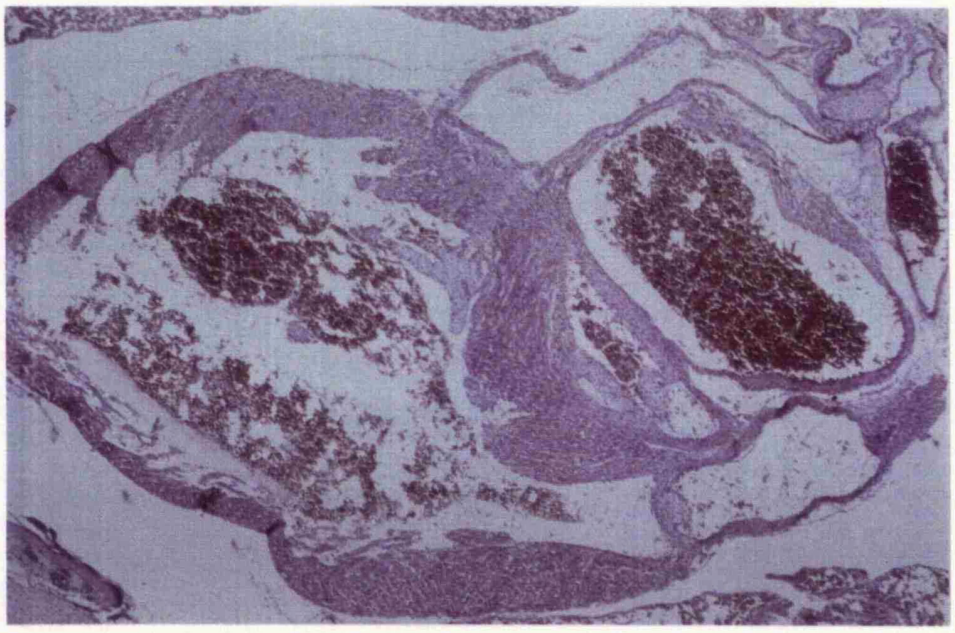


Figure 16 - Haematoxylin and eosin stained section showing a low power view of the heart from a mouse infected with coxsackie A16 virus. Magnification X 50.

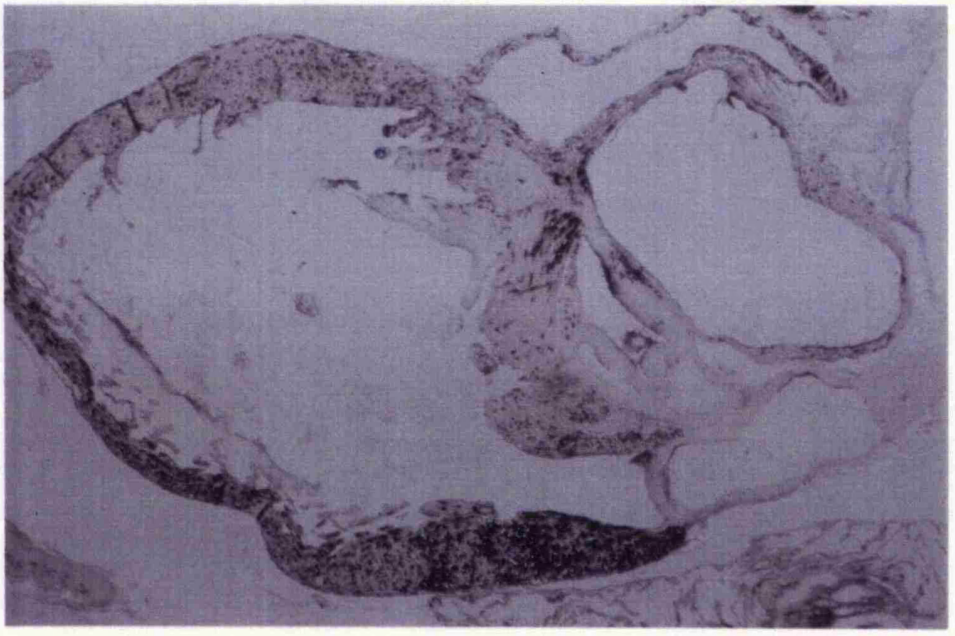


Figure 17 - *In situ* hybridisation with coxsackie virus oligonucleotides showing the presence of virus RNA widely throughout the myocardium. Magnification X 50.

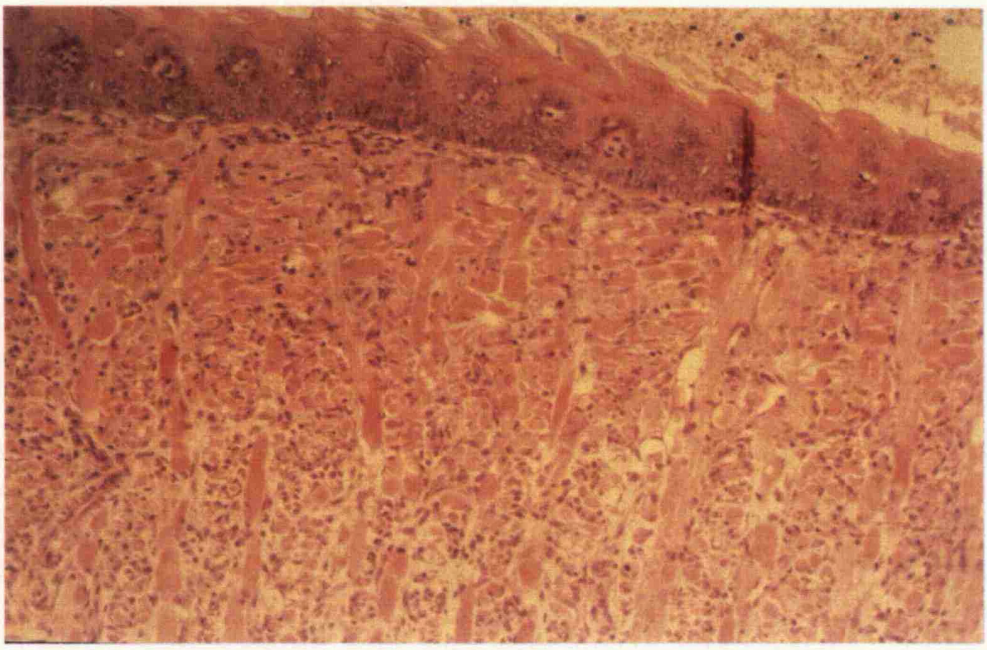


Figure 18 - Haematoxylin and eosin stained section showing the tongue from a mouse infected with coxsackie A21 virus. Magnification X 210.

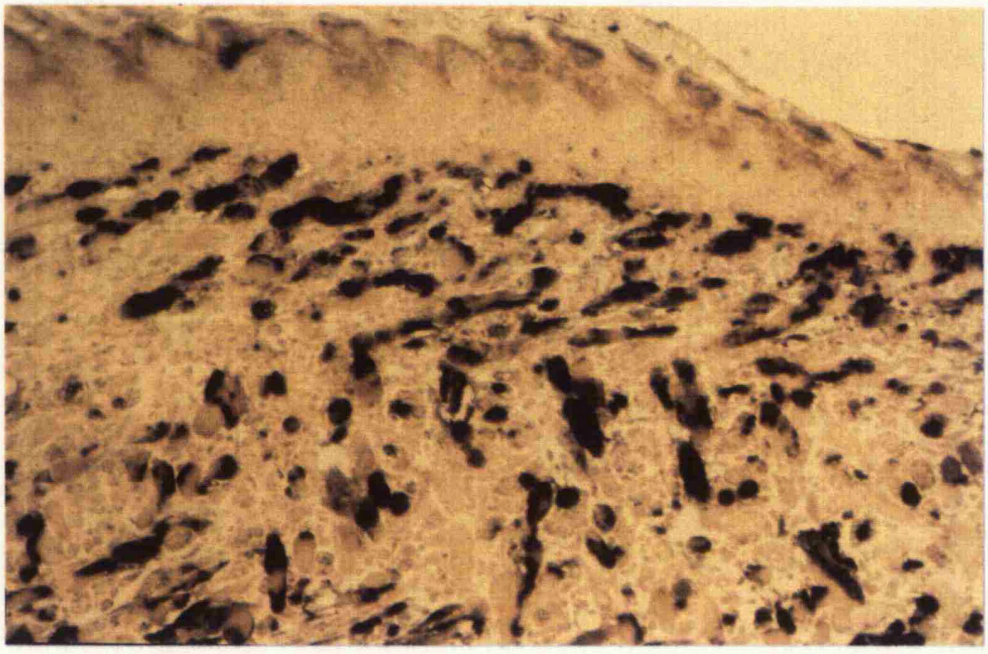


Figure 19 - *In situ* hybridisation with coxsackie virus oligonucleotides showing the presence of virus RNA in the tongue skeletal muscle fibres. Magnification X 210.

3.4.5 Strains of coxsackie virus detected

Using the cocktail of probes it was possible to detect all 11 strains of coxsackie viruses. However, from Table 2 it can be seen that one oligonucleotide (CXB6), distinguished between the coxsackie A strains from the coxsackie B strains examined.

Oligonucleotides CXB1,2,4 and 5 detected all strains except coxsackie A21, which clearly differs from the other coxsackie viruses in it's pattern of hybridisation. CXB3 appears to come from a less highly conserved region than the other oligonucleotides.

When hybridisation was repeated at higher stringency (i.e. using 50% formamide in the hybridisation buffer) all the strains except coxsackie A21 were detected.

Oligonucleotide B/ECHO did not detect any strains, even at 0% formamide, this presumably is due to the combination of a low GC content a relatively large number of mismatches. The sequences in this region may also be less highly conserved with more mismatches present in the strains used than the sequenced strains.

Table 8 - Results of ISH using individual oligonucleotides and mice infected with each strain of coxsackie virus (at 30% formamide).

Oligonucleotide	Virus										
	A5	A7	A8	A10	A16	A21	B1	B2	B3	B4	B5
CXB1	++	+	+	++	++	+	++	+	++	+	++
CXB2	++	+	+	++	++	-	++	+	++	+	++
CXB3	++	-	++	++	++	-	+	-	++	++	-
CXB4	++	++	++	++	++	++	++	++	++	++	++
CXB5	++	++	++	++	++	-	++	++	++	++	++
CXB6	-	-	-	-	-	-	+	++	++	++	++
B/ECHO	-	-	-	-	-	-	-	-	-	-	-

- no signal
+ weak signal
++ strong signal

3.4.6 Other viruses

Tissue known to contain Cytomegalovirus (small bowel), Epstein Barr virus (lymph node), Human papilloma virus (cervix) and Herpes simplex virus (brain) were also probed using the coxsackie oligonucleotides. None of these viruses were detected and RNA preservation in tissues was confirmed with mitochondrial rRNA probes. Tissue from the spinal cords of monkeys infected with 3 polio virus strains were also tested, all giving negative results with the coxsackie oligonucleotides. The reason for these latter results was not clear because no signal was seen with the mitochondrial rRNA probes (indicating poor RNA preservation) and the amount of polio virus present within the spinal cord may be too low to detect by *in situ* hybridisation.

3.4.7 Control probes

In both virus infected and control uninfected mice the presence of RNA within sections was confirmed using control probes.

Using the G3PDH oligonucleotides RNA was demonstrated predominantly in skeletal muscle, with some signal present in epithelium within the lung. Little or no signal was seen in other tissues including the heart (Figure 20).

Mitochondrial rRNA had a much wider distribution, with signal seen in all tissues. Particularly strong signal was present in skeletal and cardiac muscle and brown fat (Figure 21).

Signal with the control probes disappeared after RNase predigestion of the tissues and similar artefacts to those observed with the coxsackie probes were also observed.

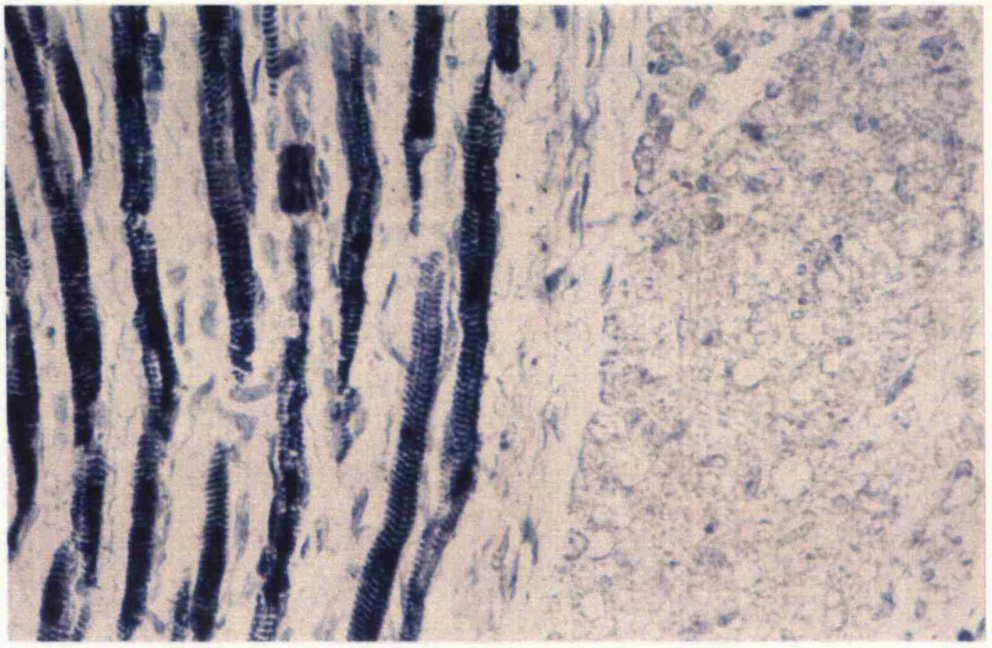


Figure 20 - *In situ* hybridisation of murine skeletal muscle and brown fat using the oligonucleotide probes to G3PDH mRNA. Magnification X 210.

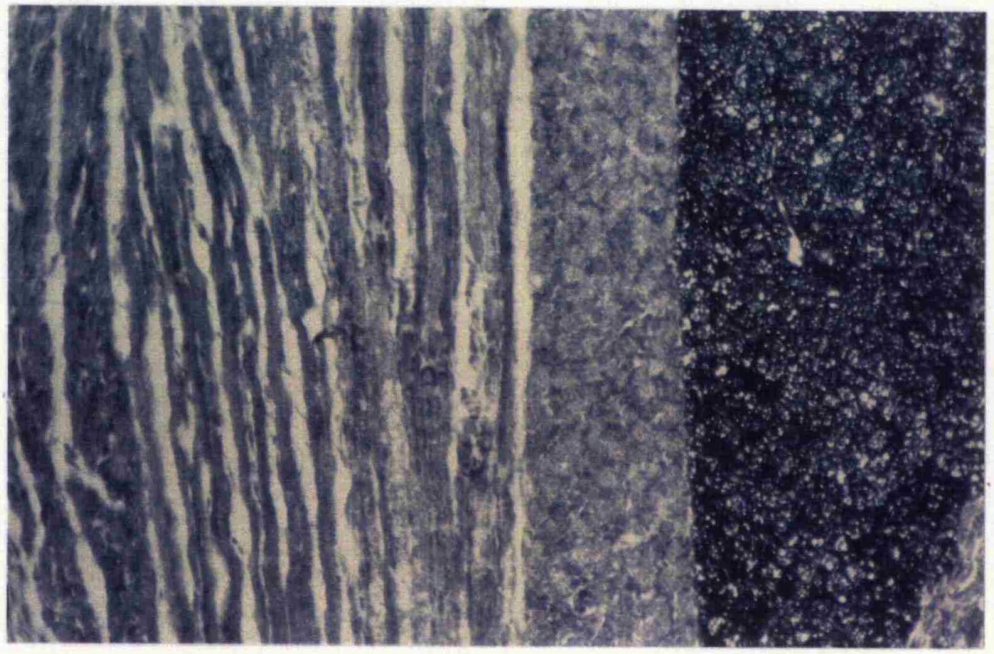


Figure 21 - *In situ* hybridisation of murine skeletal muscle and brown fat using the oligonucleotide probes to mitochondrial rRNA. Magnification X 210.

3.5 Discussion

The method used in this study allows easy detection of all coxsackie strains examined in formalin fixed tissues. The binding of probe was shown to be specific by the absence binding of a similarly constructed probe to EBV and by loss of signal after RNase treatment.

The findings using oligonucleotides separately correlates well with the published sequence data for coxsackie B viruses. The only oligonucleotide giving strong binding with coxsackie A21 virus was probe 4, which has only 3 bases mismatched with the published sequence for coxsackie A21 virus; all the other probes had 5-15 mismatched bases with the coxsackie A21 virus sequence. Under conditions of higher stringency (50% formamide) no binding to coxsackie A21 virus was seen. This data shows that a high degree of homology between probe and target sequence is necessary for hybridisation to occur, making the chance of non-specific hybridisation very small.

Using the oligonucleotides separately it also was possible to differentiate between coxsackie A or B viruses, in those strains tested, by the differential binding of oligonucleotide CXB6. This probe is unlikely to be totally group B specific because it should detect coxsackie A9 virus based on it's published sequence (Chang *et al*, 1989). The differences in probe binding to coxsackie A21 virus suggest that this strain differs significantly in it's genetic sequence to the other coxsackie viruses studied.

The distribution of viral RNA within infected mice correlated well with morphological evidence of damage, which has previously been documented (Roberts and Boyd, 1987). Two patterns of skeletal muscle involvement were seen with all 5 strains of coxsackie B virus having a much more limited distribution than the 6 coxsackie A virus strains suggesting that more than one type of skeletal muscle receptor is present for these viruses.

The presence of viral RNA in the CNS was of particular interest in the coxsackie B virus infected mice, especially in view of the suspected association with motor neuron disease (Kennedy *et al*, 1988;Bartfield *et al*, 1989). Virus RNA was detected in anterior horn cells of the cervical and thoracic spinal cord, brain stem and occasional cortical cells, confirming the tropism of coxsackie B virus for the sites affected in motor neuron disease. The mechanism of virus entry into the CNS is

unclear, it has been suggested that entry may be via motor end plates (Morrison and Fields, 1991). The absence of virus in the lower spinal cord in coxsackie B infected mice would support this hypothesis because skeletal muscle involvement was limited to the front limb girdle, head and neck regions i.e. those muscle groups supplied by the motor neurones infected. However, the involvement of occasional neurones in the upper cord and brain stem in coxsackie A16 virus infected mice would not because skeletal muscle involvement was widespread.

These results show that the oligonucleotides and ISH technique work well in fixed tissue and that the conserved regions from published sequences are also highly conserved in the strains for which sequence data are not available (and probably have some important function). It would therefore be unlikely that wild strains would differ so radically in sequence that none of the oligonucleotides would detect them by ISH.

4. *IN SITU* HYBRIDISATION OF CASES OF IIMD

4.1 Case selection

Cases were chosen by reviewing all previous muscle biopsy reports in the files of LRI, and identifying those with pathological features consistent with a diagnosis of idiopathic inflammatory muscle disease. A total of 27 cases were identified and all the case notes were retrieved from medical records for review. All the features necessary for a diagnosis of IIMD as described previously (Bohan and Peter, 1975) were looked for in the notes, in addition any other relevant information was recorded. Features such as: duration of proximal weakness, any associated clinical disease (especially autoimmune disease, lung fibrosis, malignancy or arthralgia/arthritis) and the results of serological studies for autoantibodies or virus antibodies were all noted. As the study was retrospective many of these features were not recorded in the notes.

After review of the notes and remaining muscle tissue 10 cases from the LRI were eliminated from the study. In 4 cases the remaining muscle tissue was inadequate because of the absence of the reported pathological features (this may be because these blocks have been used for other studies and areas of inflammation or necrosis may have been cut through). The other 6 cases did not have enough of the clinical and pathological features required to give a definite or probable diagnosis of IIMD (Bohan and Peter, 1975).

In addition to the cases above, all the recent muscle biopsies were reviewed from KGH, and a further 9 suitable cases of IIMD were obtained.

In total 26 suitable cases were identified, 19 had formalin fixed paraffin tissue available (cases 1,2,3,4,6,7,8,9,13,15,18-26) and 11 frozen muscle (cases 5,6,10-18).

By combining clinical and pathological features a diagnosis of probable/definite IIMD was made and this was categorised as either DM, JDM, PM or IBM on these features.

Patient details are given in the Table 9.

Table 9 - Clinical details of LRI cases (1-17) and KGH cases (18-26).

Case	age (years)	sex	CPK	EMG	duration of weakness	rash	serology	other	diag-nosis
1	31	f	>1000	myopathic	8 weeks	none	anti-Jo1 +ve	arthritis	PM
2	75	m	unknown	unknown	unknown	unknown			IBM
3	80	f	>1000	unknown	unknown	none	ANA +ve	carcinoma of the colon	PM
4	46	f	>1000	myopathic	6 weeks	none	anti-Jo1 -ve		PM
5	65	f	244	myopathic	unknown	yes	anti-nRNP +	lung fibrosis	DM
6	63	m	756	myopathic	12 months	none			PM
7	65	m	>1000	myopathic	26 weeks	none	anti-Jo1 +ve	dilated cardiomyopathy	PM
8	46	f	>1000	myopathic	unknown	none	ANA +ve	Insulin dependant diabetes MCTD	PM
9	26	m	>1000	myopathic	unknown	none	ANA +ve ENA +ve		IBM
10	46	f	817	myopathic	10 weeks	none	ANA +ve ENA +ve		PM
11	79	f	140	myopathic	4 years	none	ANA -ve		IBM
12	43	f	940	myopathic	5 weeks	none			PM
13	28	f	>3000	myopathic	4 weeks	none		post-partum	PM
14	65	f	normal	myopathic	8 weeks	yes	ENA -ve		DM
15	85	f	140	myopathic	8 weeks	yes		carcinoma of the colon	DM
16	58	m	>3000	myopathic	18 months	no			IBM
17	48	m	840	myopathic	6 weeks	no		renal failure	IBM
18	57	m	12,000	myopathic	4 weeks	yes	ANF +ve	type II diabetes	DM
19	18	f	7,400	myopathic	21 weeks	no	ANF -ve		PM
20	10	f	636	myopathic	16 weeks	yes	ANA +ve ENA -ve		JDM
21	39	f	13,000	myopathic	43 weeks	no	ANA -ve ENA +ve		PM
22	11	f	3,000	myopathic	12 weeks	yes	ANA +/- CXB IgM -ve		JDM
23	46	m	normal	myopathic	26 weeks	no	ANA +ve ENA +ve Anti-Jo-1 -ve		PM
24	56	f	834	unknown	unknown	yes	ANF +ve		DM
25	46	m	2,000	myopathic	26 weeks	no	ANA +ve ENA +ve	MCTD	PM
26	50	f	normal	myopathic	26 weeks	no	ANF -ve ENA -ve		PM

4.2 Adaptation of ISH to fresh tissue

All the ISH reactions on the mice tissues were on formalin-fixed, paraffin-embedded sections. As many of the muscle biopsies did not have any fixed tissue available a method for carrying out ISH on frozen tissue was developed.

Fixation of frozen sections

Many different fixatives are available for ISH including ethanol, acetone, formalin and paraformaldehyde. Of these cross-linking fixatives give the best results for RNA detection (Angerer *et al*, 1987). Paraformaldehyde gives rapid fixation without excessive cross-linking. In order to investigate the optimum time period for RNA preservation and detection sections of skeletal muscle were cut and mounted on to silane coated glass slides and air dried within the cryostat for 20 minutes (to ensure adhesion to the slides). The tray of slides was then immediately taken to the cold room (+4^{o}C) and dipped into 4% paraformaldehyde / 1 X PBS, for periods of either 30 seconds; 1, 3, 5, 10, 15 or 30 minutes. The slides were then placed into DEPC treated water and ISH was carried out from the prehybridisation step as described in appendix 2.

Using the oligo d(T) and mitochondrial rRNA oligonucleotides optimum signal was seen after 3-5 minutes fixation (see Figure 22). Presumably at lower fixation periods the RNA is not adequately retained and at fixation periods of 15 minutes or more the cross-linking is too excessive to allow penetration of the probes.

Figure 22 - *In situ* hybridisation of frozen sections of human skeletal muscle with oligo d(T) probes after 5 minutes fixation in 4% paraformaldehyde. Mag. X 130.

4.3 Methods of storage

In order to allow carrying out ISH on batches of cases different storage conditions were investigated.

Cryostat sections (4μm) were cut and mounted on to silane coated glass slides and air dried within the cryostat for 20 minutes. They were then fixed in 4% paraformaldehyde for either 1 or 5 minutes and transferred to 95% IMS (5 minutes) and 99% IMS (5 minutes). Sections were either kept in ethanol or rapidly air dried with an electric fan and stored under the following conditions:

(i) Room temperature (dry)
(ii) Room temperature (in ethanol)
(iii) +4°C (dry)
(iv) -20°C(dry)

n.b. Sections were not stored under ethanol in the fridge/freezer because of the risk of ignition of ethanol vapour.

After 1 week the sections were probed using oligo d(T) and mitochondrial rRNA oligonucleotides, with the standard protocol (see appendix 2), starting from the prehybridisation step.

Signal was obtained in sections stored at +4°C and -20°C, but not in those stored at room temperature. However, the intensity of signal and morphological preservation of the tissue was poor compared with sections used immediately after cutting.

I therefore decided not to use stored sections.

4.4 Choice of control probe to demonstrate RNA preservation

Although I had designed oligonucleotides to G3PDH mRNA for use as control probes, a number of other probes were available within the department:

(i) oligonucleotides to mitochondrial rRNA sequences (Chester *et al*, 1990).
(ii) oligo d(T) probe (Pringle *et al*, 1989).
(iii) oligonucleotides to kappa and lambda light chain mRNA (Pringle *et al*, 1990).

All these probes were tried on the first 10 cases of IIMD and all worked to some degree. The G3PDH produced the weakest signal, even with very long substrate development times (20 hours). Signal was present diffusely throughout the cytoplasm, but a clear demonstration of type II fibre predominance was only seen in the frozen and optimally fixed rat skeletal muscle (Figures 23 and 24). The weakness of the signal and long development times meant that it was sometimes difficult to differentiate signal from background. Mitochondrial rRNA was demonstrated as punctate signal, predominantly in type I fibres, and often around the periphery of myofibres (Figure 26). Each focus of signal may represent a single mitochondrion, using the con-focal laser microscope and Fast-Red development of the alkaline phosphatase, these foci measured 0.5 μm in length, which is about the size of a mitochondrion. Kappa and lambda mRNA produced strong signal within plasma cells (Figure 27), but unfortunately these were only present in about 20% of cases of IIMD, so that these probes were not suitable for this study. Oligo d(T) produced weak-moderate signal around the periphery of myofibres, which was diffuse in nature. A further disadvantage with the oligo d(T) probe is that formamide cannot be used at any stage during ISH (because of the low melt temperature of A-T rich hybrids) so that a different set of hybridisation buffers and post-hybridisation washes had to be prepared.

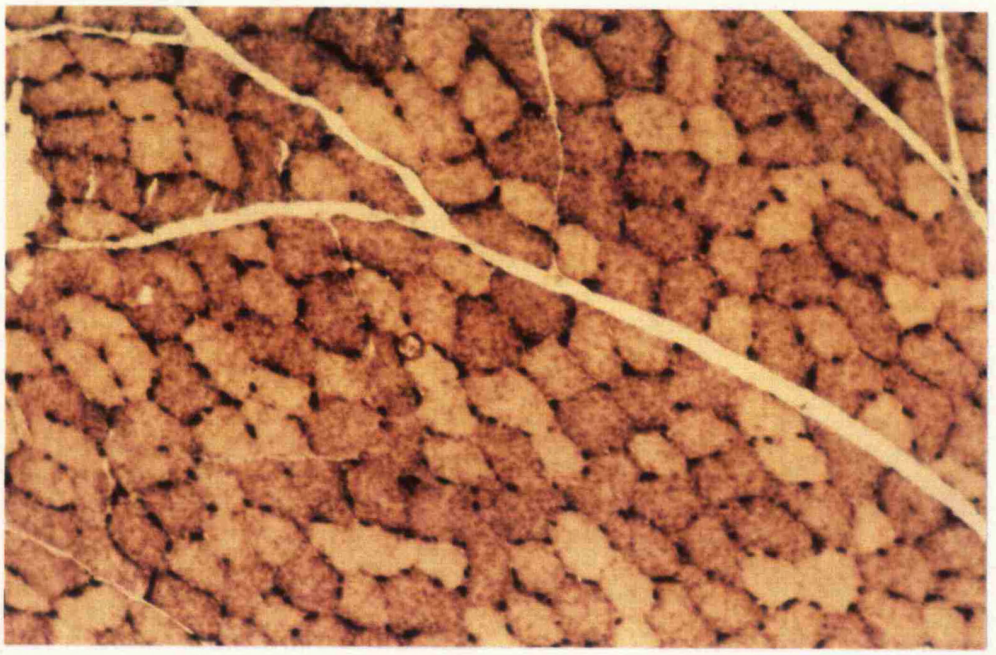


Figure 23 - *In situ* hybridisation of rat frozen sections with oligonucleotides to G3PDH mRNA showing signal within type II fibres. Magnification X 130.

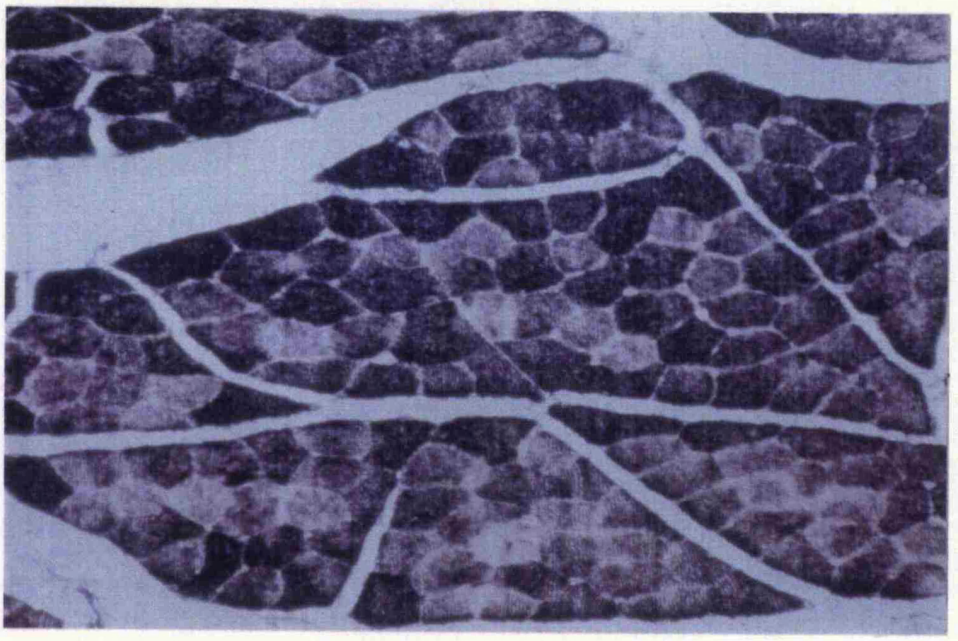


Figure 24 - *In situ* hybridisation of formalin fixed sections of rat skeletal muscle with oligonucleotides to G3PDH mRNA. MagnificationX 130.

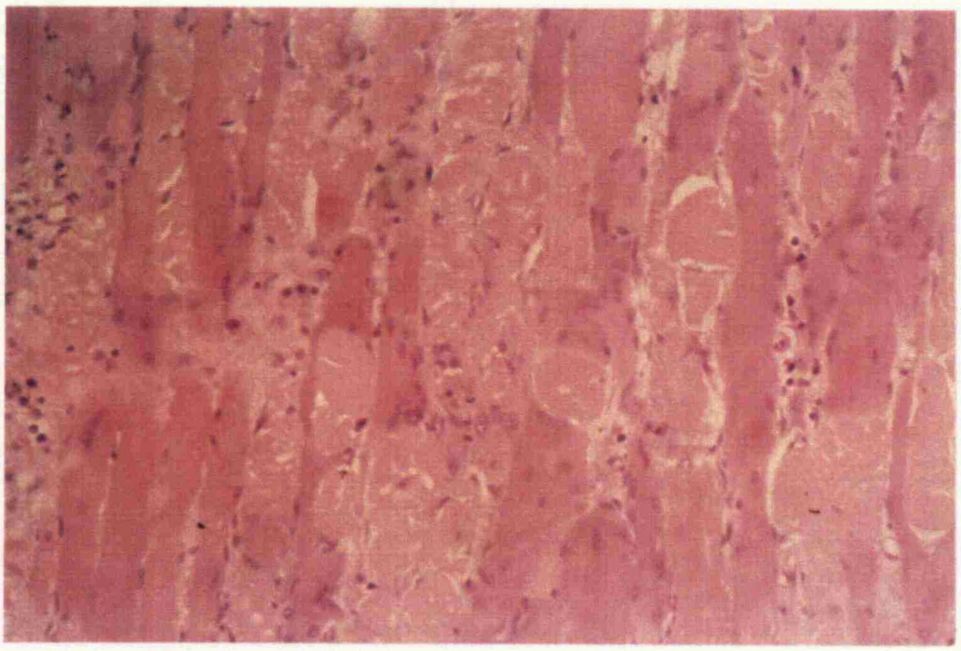


Figure 25 - Haematoxylin and eosin stained section from case 25 (polymyositis) showing fibre necrosis, regeneration and inflammatory cell infiltrate. Magnification X 130.

Figure 26 - *In situ* hybridisation from case 25 with oligonucleotides to mitochondrial rRNA showing RNA preservation within myofibres, inflammatory cells and blood vessel walls. No signal is seen in necrotic segments of myofibres. Magnification X 130.

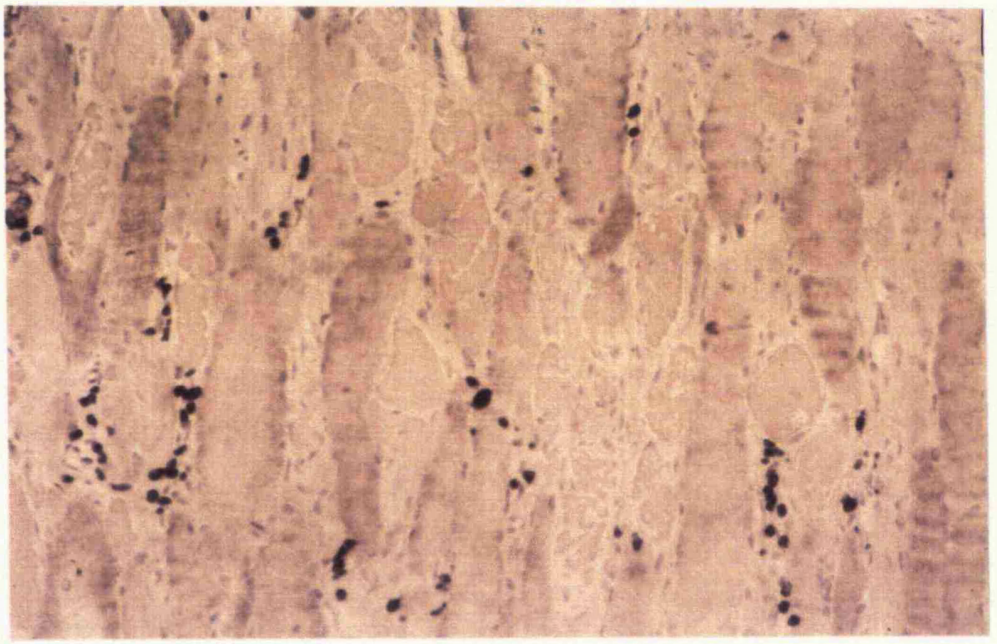


Figure 27 - *In situ* hybridisation from case 25 with oligonucleotides to Kappa light chain mRNA showing mRNA preservation within plasma cells. Magnification X 130.

In view of the discrete strong signal produced with the mitochondrial rRNA oligonucleotides and because they could be used at the same formamide concentration as the coxsackie oligonucleotides, I decided to use these as a measure of RNA preservation within muscle.

4.5 ISH on IIMD cases using mitochondrial rRNA probes

All the cases showed evidence of rRNA within sections. Signal was stronger in the type I muscle fibres and particularly strong in regenerating fibres. Signal was also present within inflammatory cells and blood vessels. In large blocks of tissue (i.e. the open muscle biopsies) the signal was strongest around the edges of the biopsy, presumably due to more rapid penetration of fixative. Signal was not seen in sections pretreated with RNase (except the artefactual signal described in section 4.6.1).

4.6 ISH on IIMD cases using coxsackie probes

Of the 30 cases examined, 5 showed positive signal within myofibres and 9 within interstitial mononuclear inflammatory cells. The signal was not seen if probe was omitted from the hybridisation buffer.

4.6.1 Signal within myofibres

Of the 30 cases examined, 5 showed strong signal using the coxsackie probes within myofibres. The signal was focal and often localised near vacuoles and nuclei (Figure 29). On review of these positive cases it was noted that all 5 cases showed the characteristic histological features of inclusion body myositis, with signal in the same areas as the filamentous structures seen by electron microscopy (Figure 50) (which had previously been thought by some to represent viral structures - Chou, 1967). However, on further investigation this signal was found to be an artefact, rather than genuine hybridisation. Although no signal was present if the probe was omitted, showing that it was not due to some other component of the of the ISH procedure, such as binding of the anti-digoxigenin antibody, the myofibre signal persisted after RNase pretreatment (see appendix 2) of the sections indicating that it was not due to hybridisation to RNA (unlike the signal with control probes which completely disappeared).

An artefact of this type had not been encountered previously within the department and warranted further investigation. Most possible types of non-specific binding of probe should be blocked by the usual conditions employed during hybridisation:

(i) The salt, temperature and formamide are calculated to provide optimum conditions for specific hybridisation.
(ii)The sonicated salmon sperm DNA, which is denatured immediately prior to use, should block any non-specific DNA binding sites within the tissue prior to the addition of probe.
(iii)Dextran sulphate, as well as acting as an accelerator, is negatively charged so blocks positive sites within the tissue.

In addition to the blocks of muscle, in 2 cases (cases 20 and 24) the skin biopsies were also assessed for RNA preservation and the presence of virus. In both of these cases mitochondrial rRNA preservation was demonstrated but there was not any signal in the skin with coxsackie probes.

4.6.2 Further investigation of non-specific signal

Confirmation that signal was not due to hybridisation

A number of other oligonucleotides available within the department were also used, including ones directed against mRNA sequences to insulin, kappa light chain, parathyroid hormone related peptide and to the small nuclear RNA coded by Epstein-Barr virus. All these latter oligonucleotides produced identical signal within myofibres. Therefore the signal could not be due to any form of specific hybridisation by the probes.

Was the signal due to binding of the digoxigenin label of the oligonucleotides?

In order to investigate this possibility attempts to block signal with unincorporated digoxigenin-11-dUTP was carried out, but this made no difference. The EBV oligonucleotides were labelled using biotin-dUTP in the same homopolymer tailing method used for digoxigenin labelling (see appendix 2). These biotin labelled oligonucleotides were then used for ISH on the muscle (with a case of Hodgkin's disease lymph node as a positive control). ISH was tried out as before, but a different detection method was employed to visualise the biotin label (streptavidin 1:2000 in TBS, followed by biotinylated alkaline phosphatase 1:600 in TBS) with the usual NBT/BCIP in buffer 3 development.

Although this produced high levels of background the same artefactual signal was present. Also an FITC labelled oligonucleotide was similarly employed with similar results.

The artefact was therefore unlikely to be due to due to the digoxigenin label.

Was the signal a charge related phenomenon?

Oligonucleotides have a negative charge (mainly related to the phosphate groups in the sugar-phosphate backbone), so that if the blocking action of molecules in the hybridisation buffer were ineffective, as seen with the keratin signal in the mice, probes could bind to positively charged molecules in the tissue section.

Attempts to block the positively charged sites in the tissue with by acetylation, either using the published method (Hayashi *et al*, 1978) or in a non-aqueous medium which effectively blocked the keratin signal, was unsuccessful, even after immersing the slides for up to 1 hour in 5% acetic anhydride.

Another negatively charged blocking agent was also tried, heparin sulphate, by adding either a 1% or 5% concentration to the prehybridisation and hybridisation solutions. This too was ineffective.

In an attempt to block the negative charge on the probe a basic amino acid (lysine) was added to the hybridisation buffers at 0.1M or 1M concentrations, but this too was ineffective.

Other attempts to block myocyte signal.

The concentration of salmon sperm DNA in the prehybridisation and hybridisation buffers was increased 3 x normal (to 450ug/ml), and also the salmon sperm DNA was given an extra 30 bursts of sonication to further fragment it, but neither of these methods was effective.

Also both unlabelled oligonucleotide and biotinylated oligonucleotide were added to the prehybridisation and hybridisation buffers at 10 x the concentration of digoxigenin labelled probe, but the artefact persisted.

Hydrolysis of the sections (by immersion in 0.2N HCl after dewaxing) made no difference.

Adding bovine serum albumin at either 0.1% or 5% to the prehybridisation and hybridisation buffers was unhelpful.

As the binding did not appear to be mediated by charge, attempts to interfere with hydrogen bonds with urea (0.1M, 1M or 2M) in the prehybridisation and hybridisation buffers was tried without success.

DNase 1 (Sigma) predigestion of tissue sections did not affect signal so that it was not mediated by binding to single stranded DNA. Also use of a non-specific protease (Pronase E - Sigma) did not remove the binding so that it is unlikely to be due to a protein effect.

The only time that the signal did disappear was at very low probe concentrations (about 0.02 ng/μl).

Increasing the proteinase K concentrations was also unhelpful.

4.6.3 Signal within mononuclear cells

After the initial ISH of all the IIMD cases for coxsackie viruses, which was effectively negative the cases were repeated using the probes at double strength (0.2 ng/μl) in an attempt to detect low copy numbers of virus. This resulted in a granular signal within the cytoplasm of mononuclear cells in the inflammatory infiltrate (Figures 30 and 31). This signal was also resistant to RNase pretreatment (see appendix 2) and was seen with oligonucleotides directed against EBV.

On examination of the H&E sections of these cases it was noted that the positive cells had a similar size, shape and distribution to mast cells (Figure 32). Mast cells, like keratin are rich in basic substances (e.g. histamine) and acetylation (see appendix 2) abolished this signal, without abolishing the signal with the control RNA probes.

A similar signal to this mast cell binding has been reported previously with probes directed against TMEV (Rosenberg *et al*, 1989). This was interpreted by the authors as evidence of a "novel picornavirus in human dermatomyositis", despite the virus not being known as a human pathogen. Also more recently others have demonstrated binding of coxsackie probes to mononuclear cells in IIMD (Behan *et al* ,1992), and interpreted this as evidence of virus presence.

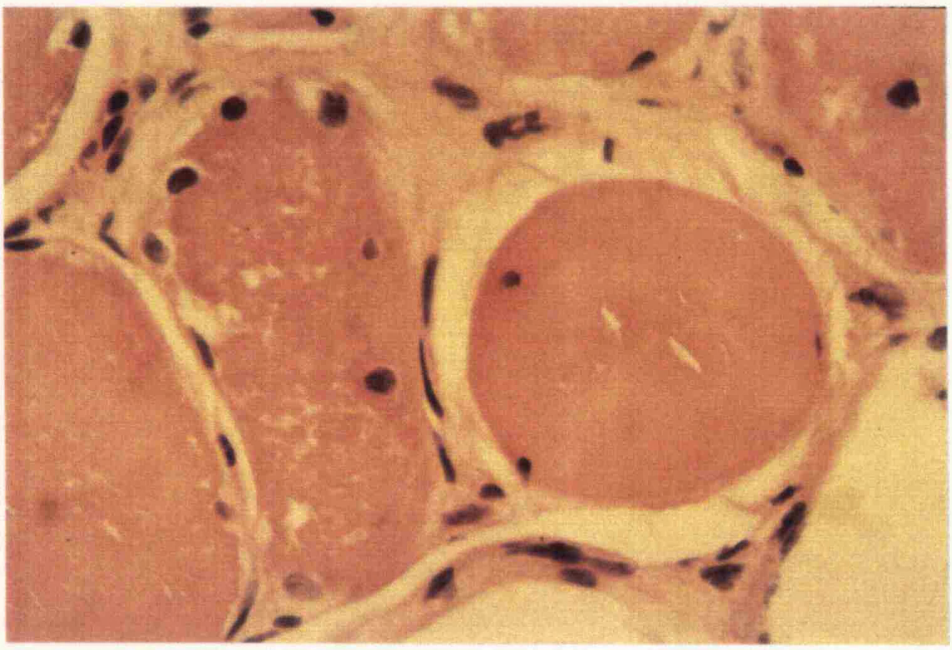


Figure 28 - Haematoxylin and eosin stained paraffin section from case 2 (inclusion body myositis) showing a vacuolated fibre. Magnification X 840.

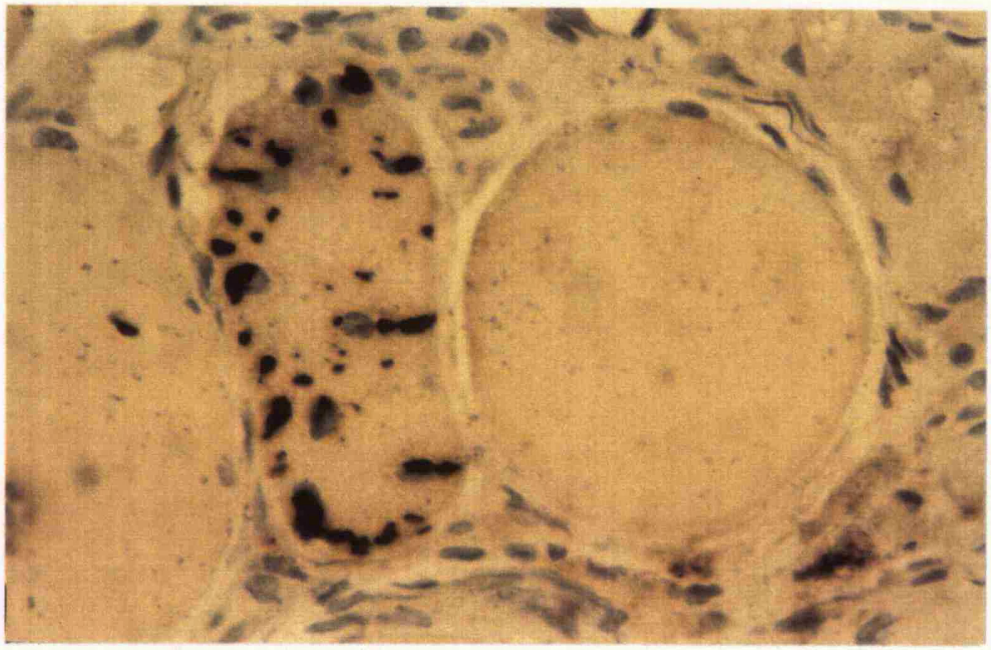


Figure 29 - *In situ* hybridisation, following RNase pretreatment of sections, from a case 2, with oligonucleotides to coxsackie virus RNA, showing discrete signal within muscle fibres, particularly near to vacuoles and within nuclei. Mag. X 840.

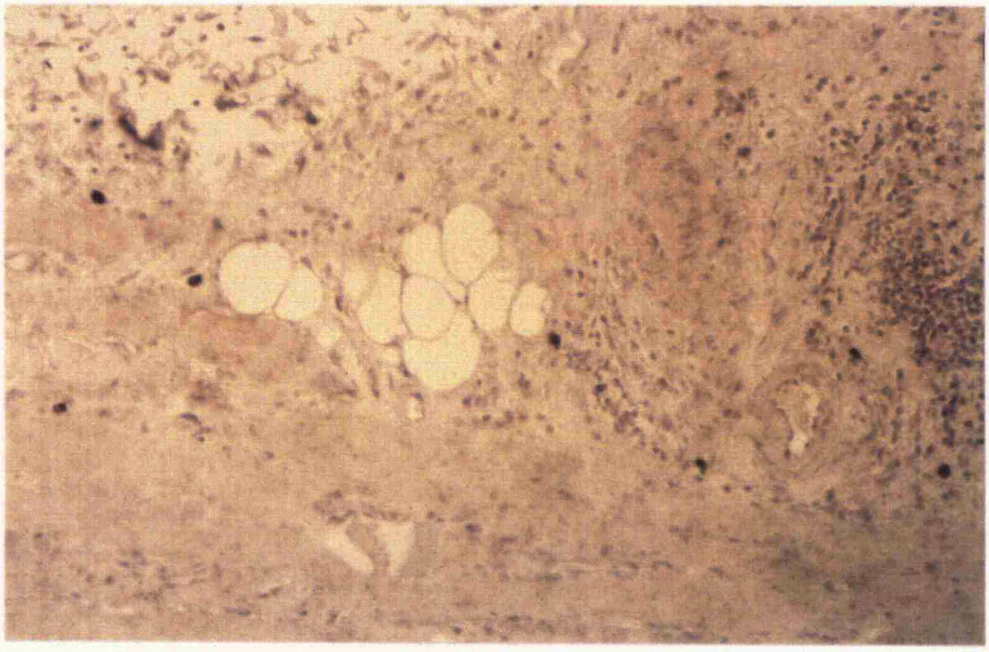


Figure 30 - *In situ* hybridisation of case of IIMD (case 22) with oligonucleotides to coxsackie virus RNA showing signal within interstitial mononuclear inflammatory cells. Magnification X 130.

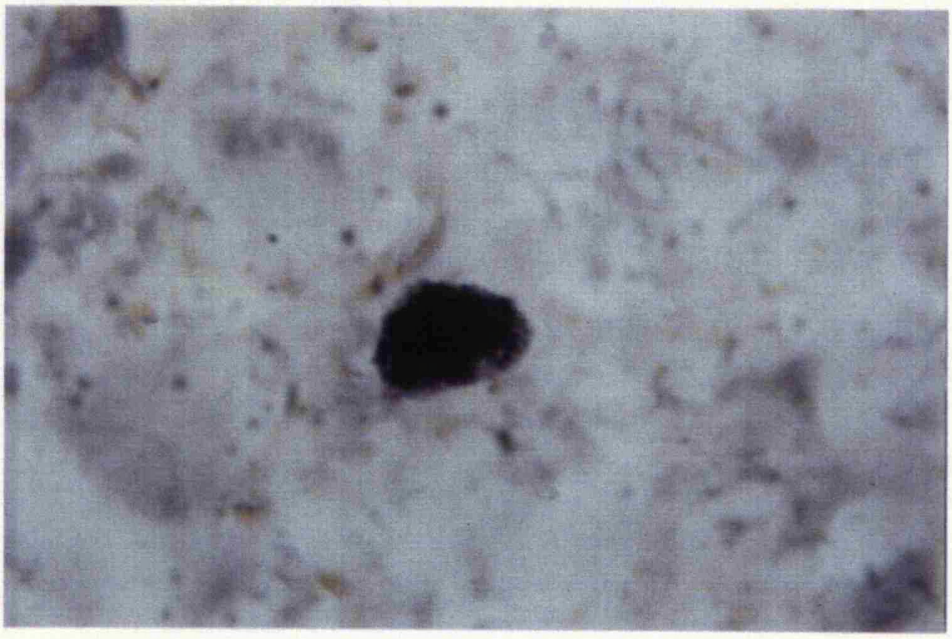


Figure 31 - High power of probe binding to interstitial cells showing granular pattern. Magnification X 1680.

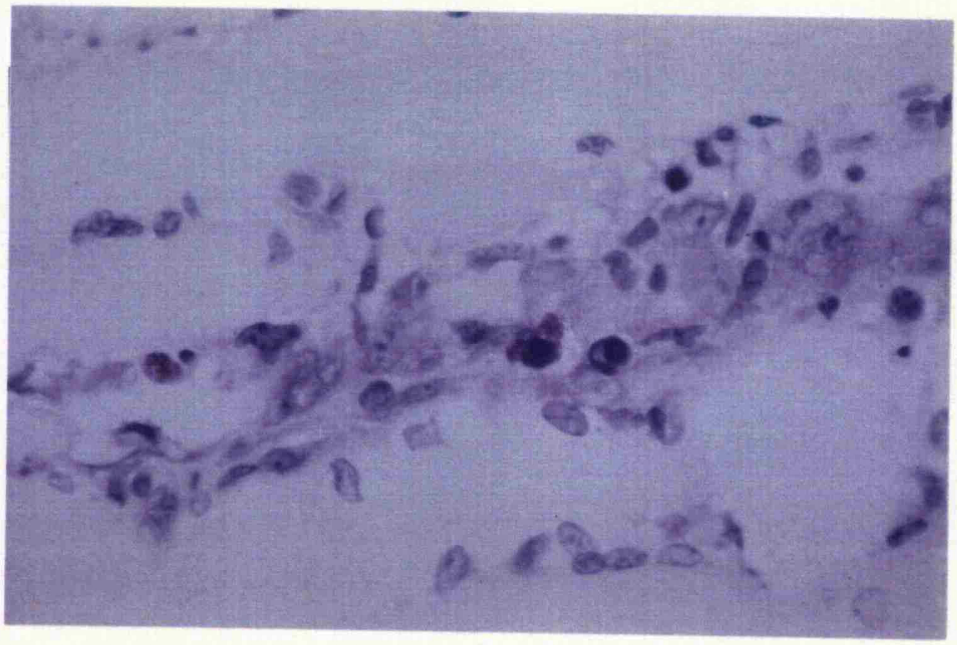


Figure 32 - Parallel toluidine blue stained section from case 22 showing metachromatic granules within mast cells in a similar distribution to that of probe binding. Magnification X 840.

4.6.4 Effect of denaturation of tissue sections

The cases were repeated with denaturation of the tissue sections. This was done because it has been suggested that equal amounts of positive and negative stranded RNA can be produced in coxsackie infected muscle (Cunningham *et al*, 1990), and therefore it was possible that the positive and negative strands would hybridise to form double stranded RNA.

Denaturation was carried out by heating the sections to 100°C for 15 minutes in Terasaki chambers, once the hybridisation solution (including probe) had been added to the sections. The sections were then hybridised as usual overnight at 37°C.

Denaturation did not affect morphology, background or produce positive results!

4.7 Discussion

These ISH results do not support the hypothesis that coxsackie virus RNA is present in muscle from IIMD.

The non-specific binding of probe to mononuclear cells appears to be a charge related phenomenon, and is remarkably similar to the findings of others (Behan *et al*, 1992; Rosenberg *et al*, 1989), where it was claimed to be genuine hybridisation.

All cases showed RNA preservation with control probes, therefore the negative findings could not be explained by loss of RNA. Alternative explanations include the absence of virus, too little virus to detect by ISH and the possibility of wild types having a different genome to the sequenced and reference strains.

The later PCR part of the investigation would be able to detect small amounts of virus, but to exclude the possibility that wild strains of virus were not detected by these probes a number of human cases of myocarditis were also examined.

5. ISH ON HUMAN MYOCARDITIS

5.1 Introduction

In order to demonstrate the effectiveness of the ISH technique developed for coxsackie viruses I wanted to demonstrate coxsackie virus RNA in infected human tissue. The only condition where the virus has been convincingly been associated with human disease is acute myocarditis. Although human myocarditis has been associated with a number of viruses, the most commonly implicated is the coxsackie group (see reviews: Leslie *et al*, 1989; Woodruff, 1980). Several recent studies have also claimed to have demonstrated the presence of enteroviral RNA in myocardial tissue by hybridisation studies (Bowles *et al*, 1986; Easton and Eglin, 1988; Jin *et al*, 1990; Weiss *et al*, 1991; Tracy *et al*, 1990).

As myocarditis is fairly uncommon I approached Dr.S.Variend at The Children's Hospital, Sheffield, for some cases, because of his interest in the condition. He kindly provided 10 cases, the details of which are given below.

5.2 Cases

All cases studied were retrieved from the post-mortem files and died between 1974 and 1992. Details of the cases (all of which filled standard criteria for the diagnosis of myocarditis - Aretz *et al*, 1987) and controls are given in Tables 10 and 11.

Table 10 - myocarditis cases

Case	Sex	Age	Duration of symptoms	Viral studies	
1.	F	7 years	7 days	EM heart	-ve
				Culture heart	-ve
2.	M	6 months	sudden death	EM faeces	-ve
				Serology	-ve
3.	M	7 months	sudden death	EM faeces	-ve
				Culture faeces	-ve
4.	F	6 days	2 days	EM faeces	-ve
				Culture faeces	+ve[a]
5.	M	18 months	2 days	EM faeces	-ve
				Culture faeces	-ve[b]
6.	M	9 months	10 days	Culture faeces	-ve
7.	M	9 days	1 day	Culture heart	-ve
				Culture faeces	-ve
8.	M	12 years	sudden death	Not done[c]	
9.	M	1 year	2 days	EM faeces	+ve[d]
				Culture faeces	-ve
10.	F	1 year	1 day	Culture faeces	-ve
				Serology	-ve

a. Coxsackie B2 virus cultured.
b. Varicella zoster IgM positive. Skin rash typical of chicken pox.
c. Death was due to head injury and myocarditis was not suspected macroscopically
d. Occasional enterovirus-like particles seen.

5.3 Method

ISH was carried out as before (see appendix 2), using the cocktail of coxsackie oligonucleotides and the mitochondrial rRNA probes to confirm RNA preservation. The cases were also examined by ISH using the probes to G3PDH.

To confirm RNA hybridisation, control sections were treated with RNase A (see appendix 2). To show specificity of hybridisation oligonucleotide probes to EBV (Pringle *et al*, 1992) were used.

Table 11 - control myocardium cases

Case	Sex	Age	Diagnosis
11.	F	9 months	Respiratory syncytial virus pneumonia
12.	M	9 weeks	Congenital heart disease
13.	F	8 months	Pneumonia
14.	M	10 weeks	Downs syndrome
15.	M	3 weeks	Congenital heart disease
16.	F	3 weeks	Pneumonia
17.	M	10 months	Septicaemia
18.	M	5 years	Congenital cirrhosis
19.	M	6 years	Alpers syndrome
20.	F	6 years	Alpers syndrome.

5.4 Results

Two cases were positive with the coxsackie probes by ISH (cases 4 and 7). The specificity was confirmed by absence of any signal with a similarly constructed and labelled probe to EBV and the loss of signal after RNase predigestion. In both cases the signal was confined to myocytes and was never seen in inflammatory cells. The infected myocytes were predominantly in areas of inflammation and immediately adjacent to obviously necrotic fibres (Figure 34). Occasionally signal was seen in single myocardial fibres without any obvious inflammation or necrosis present. This latter pattern may represent the earliest stage of infection. In one case (number 4) there was a striking sub-endocardial pattern to the distribution of virus (Figure 37). The intensity of signal in both positive cases was very strong, even in areas where there was little or no evidence of mitochondrial rRNA preservation in either infected or adjacent cells, suggesting that the viral RNA is better preserved that host RNA.

Both positive cases were further investigated using single coxsackie oligonucleotides, CXB5 and CXB6. Positive signal was seen in both cases with CXB5, but only in case 4 with CXB6. These findings suggest that in case 7 the virus may have been a coxsackie A virus rather than a coxsackie B virus.

By ISH all cases had mitochondrial rRNA demonstrable, although this was very variable, with some cases showing signal predominantly around the edge of the sections (presumably the areas fixed most rapidly). In none of the cases was G3PDH mRNA detectable, which was not surprising because it was not seen in the optimally preserved sections of mouse, within the myocardium.

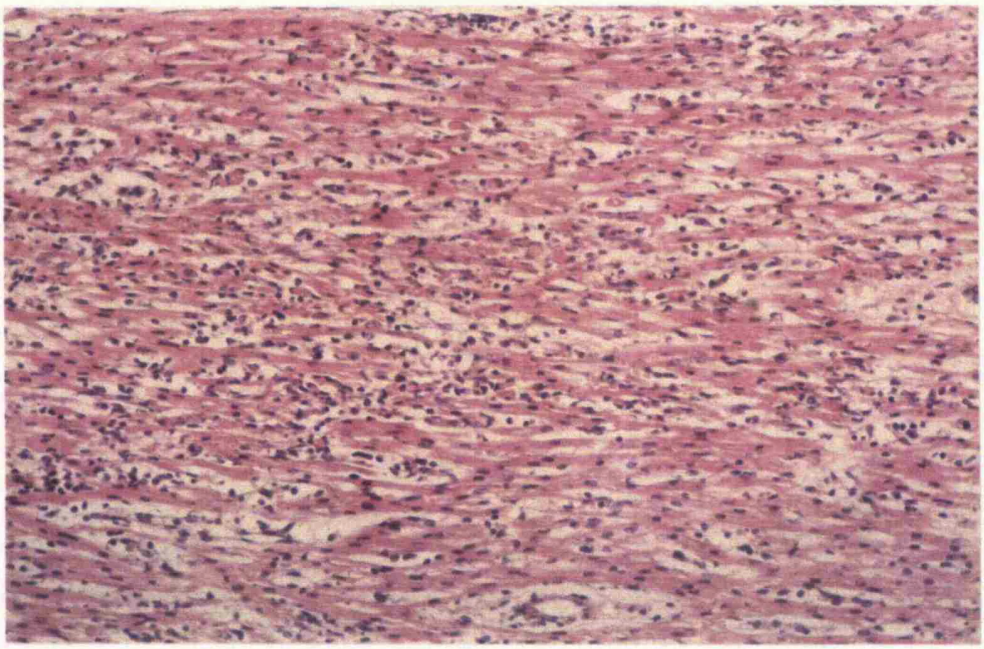


Figure 33 - Haematoxylin and eosin stained paraffin section from case 7 showing myocardial necrosis and inflammation. Magnification X 130.

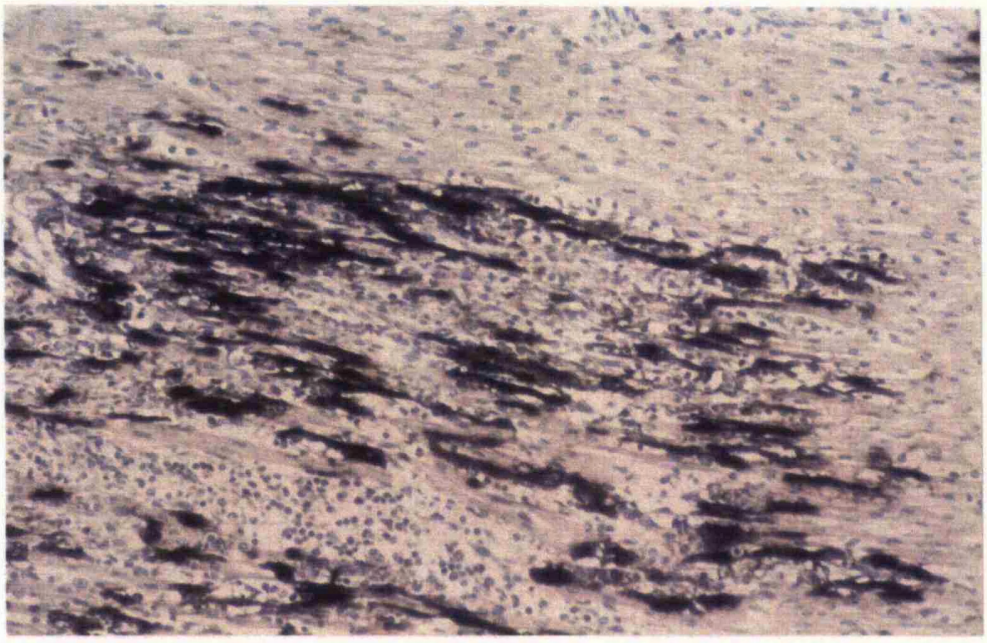


Figure 34 - Parallel section from case 7 following ISH with coxsackie virus oligonucleotides showing virus RNA within myocardial cells immediately adjacent to necrotic fibres in areas of inflammation. Magnification X 130.

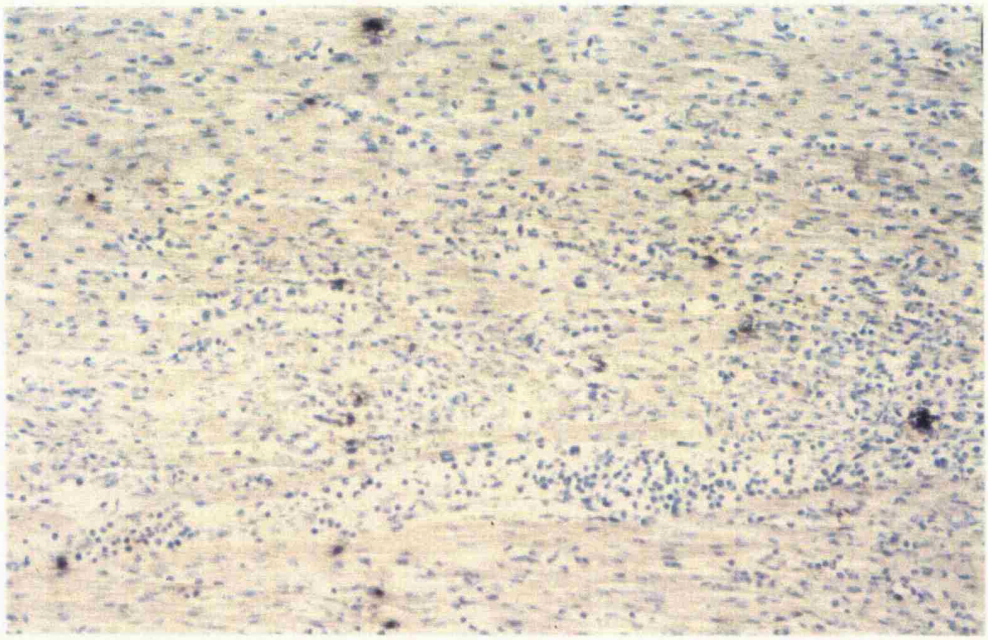


Figure 35 - Parallel section from case 7 following RNase pretreatment showing absence of ISH signal with coxsackie virus oligonucleotides. Magnification X 130.

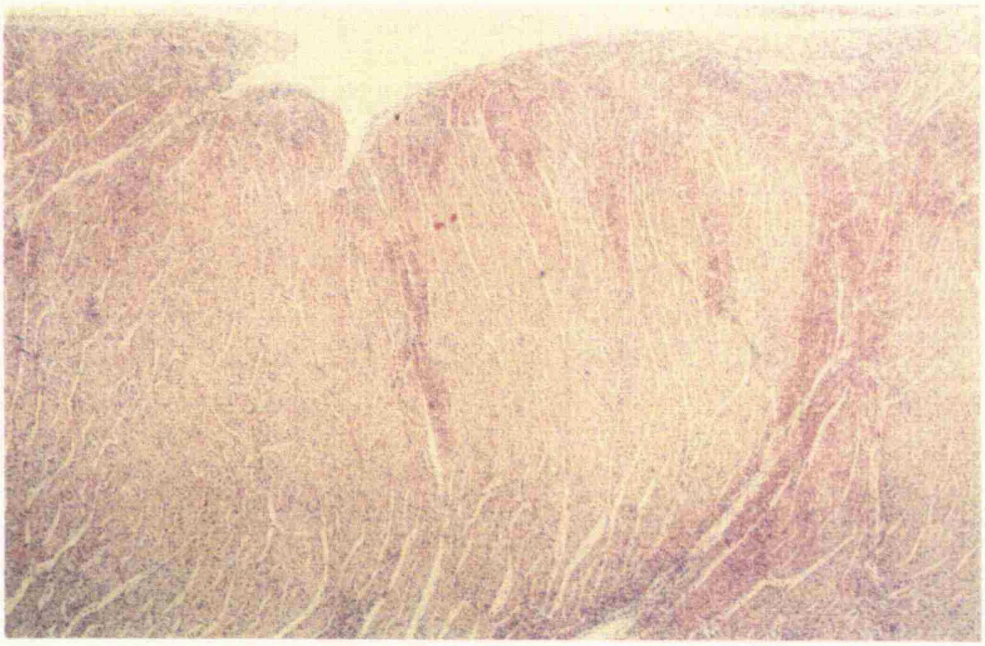


Figure 36 - Haematoxylin and eosin stained paraffin section from case 4 showing myocardial necrosis and inflammation predominantly in a subendocardial distribution. Magnification X 25.

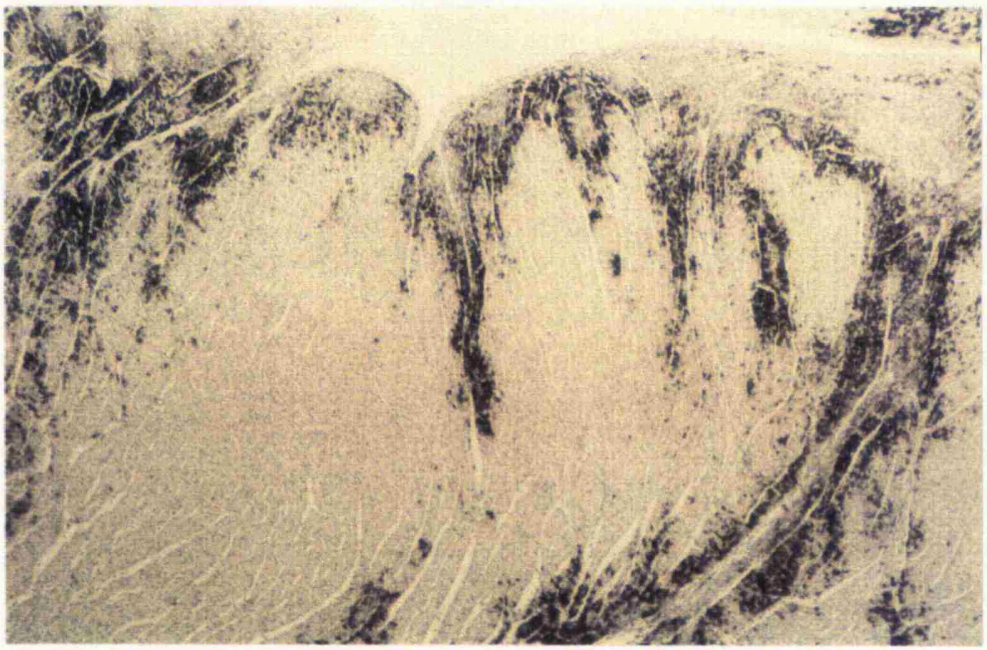


Figure 37 - Parallel section from case 4 showing the results of ISH with the coxsackie virus oligonucleotides. Magnification X 25.

5.5 Discussion

This study has demonstrated the presence of coxsackie virus RNA in archival human tissue by ISH.

The finding of 2 (20%) positive cases is in keeping with other well controlled studies on human myocarditis (Tracy *et al*, 1990; Kandolf, 1988), although these studies have been on adults. There was no obvious histological difference in the extent of necrosis or degree and nature of the inflammatory infiltrate between positive and negative cases, suggesting that the basic pathological process is the same in all these cases. Both positive cases were less than 10 days of age at the time of death, infection in these cases is likely to have occurred at or around the time of birth.

The presence of virus even in areas of poor mitochondrial rRNA preservation indicates that viral RNA is better preserved than host RNA. This may be in part due to protection from endogenous RNases by the protein capsid of mature virions.

The detection of viral RNA solely within muscle fibres and predominantly in areas of inflammation is consistent with myocarditis being caused by a direct lytic infection with virus, rather than an autoimmune mechanism (Huber, 1992; Rose *et al*, 1988), in these positive cases. The mechanism of myocardial damage in the negative cases may be due to direct infection with other viruses, such as influenza viruses, cytomegalovirus, or adenoviruses (Woodruff, 1980). However, this remains to be determined. The ISH results using the EBV probes make this virus an unlikely candidate.

The striking subendocardial distribution of viral RNA (and myocardial fibre necrosis) in case 4 was of interest as an association between endocardial fibroelastosis and coxsackie virus has been described previously (Fruhling *et al*, 1962). In this latter study coxsackie virus could be cultured in 13/28 cases of endocardial fibroelastosis (Fruhling *et al*, 1962). The findings in case 4 add further evidence in support of an aetiological association between endocardial fibroelastosis and coxsackie virus infection by confirming a viral tropism for this location within the heart.

The heart from the second positive case (case 7) showed left ventricular hypoplasia, which is also consistent with the previous suggestion of a link between coxsackie

virus infection and hypoplastic left heart (Fruhling *et al*, 1962). In this case no virus could be cultured from either the heart, kidney, liver or stool, which is surprising because of the large amounts detected by ISH within the myocardium. However, this could be explained by infection with a coxsackie A virus strain, some of which are difficult to culture (see section 3.5).

As well as providing interesting new data on human heart disease this study has clearly proved the efficacy of the ISH technique for demonstrating "wild type" virus RNA presence, even in routinely processed post-mortem tissue where RNA preservation is likely to be worse than surgical material, due to the delay in fixation.

It is therefore highly unlikely that coxsackie viruses cause human IIMD by a lytic infection of skeletal muscle in a similar way to myocarditis or murine myositis.

PART 3

POLYMERASE CHAIN REACTION STUDY

1. GENERAL PRINCIPLES

1.1 Introduction

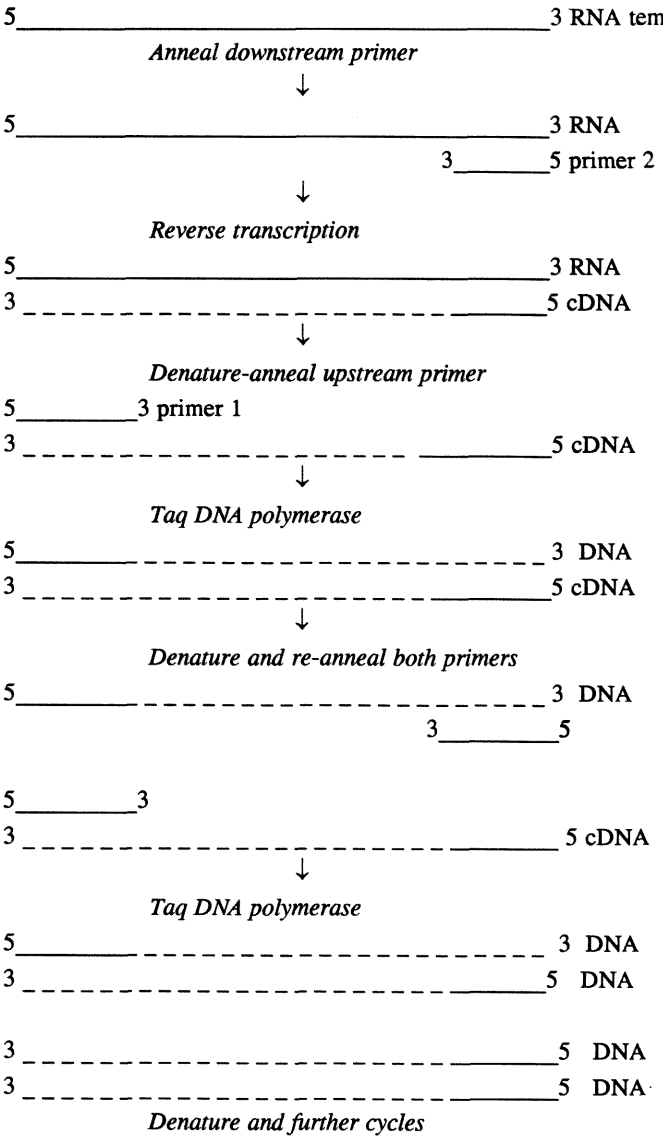
The polymerase chain reaction (PCR) is widely used as a sensitive method of detection of nucleic acid, either DNA or RNA, in a variety of situations, capable of detecting as little as one molecule of nucleic acid (Li *et al*, 1988). The technique was first described in 1985 (Saiki *et al*, 1985) where it was used for the amplification of human -globin DNA and to the prenatal diagnosis of sickle cell anaemia. The reaction was catalysed by the Klenow fragment of Escherichia coli DNA polymerase 1 which is inactivated by the heat denaturation step, so requiring fresh enzyme to be added after each cycle. The subsequent use of heat stable Taq DNA polymerase (Saiki *et al*, 1988) and the development of programmable thermal cyclers has allowed automation of PCR.

The PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridise to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. The primer extension products synthesised in one cycle can act as templates for the next, thus the number of target DNA doubles for each cycle, leading to a yield of about a million-fold after 20 cycles.

Amplification of RNA requires an initial step to convert the RNA into complimentary DNA using reverse transcriptase, because the thermostable Taq DNA polymerase will not work on an RNA template, a method first used in 1987 (Veres *et al*, 1987). The reverse transcriptase enzyme is usually derived from avian myeloblastosis virus (AMV) or moloney murine leukaemia virus (MoMuLV). A summary of the method is given in the diagram overleaf.

The technique of PCR has gained a number of applications, including diagnosis of genetic disorders, detection of nucleic acid sequences from pathogenic organisms in clinical specimens, genetic identification from forensic samples, analysis of mutations in oncogenes, generation of specific sequences for use as probes, generation of libraries of cDNA from small amounts of mRNA and the synthesis of large amounts of DNA (for reviews see Rapley *et al*, 1992; Love and Nicoll, 1992; Erlich *et al*, 1991).

Summary of reverse transcription - PCR.



1.2 Precautions taken for the PCR

The PCR is capable of amplifying as little as one molecule of DNA, so precautions must be taken to avoid contamination with trace amounts of DNA which could act as templates, particular care must be taken with the PCR product, which in positive cases can contain many millions of DNA copies.

The PCR should be carried out in a laminar flow hood, ideally with ultraviolet lights (which should be turned on when the hood is not in use). A set of pipettes dedicated for PCR, gloves, and a microfuge should be kept within the hood. Positive displacement pipettes with disposable tips and plungers may help avoid contamination from pipette barrels. All reagents, pipette tips and eppendorf tubes should be autoclaved prior to use (Sambrook *et al*, 1989).

Fresh gloves should be put on when beginning work in the PCR area, and these should be changed frequently.

All reagents should be prepared in small aliquots and not used for other purposes. New glassware should be used.

When preparing the reaction in the microfuge tube add the template DNA last, taking care not to create aerosols, keeping the tops of tubes not in use closed. Gloves should be changed after this step.

The positive control should be prepared ahead of time in a different area of the laboratory, using the lowest possible dilution of template. It is also necessary to include a no template negative control.

1.3 Components of the PCR

1.3.1 Oligonucleotides

Oligonucleotides for use as primers in the PCR are usually about 20 nucleotides in length. These are too short to form stable hybrids at the temperature used for polymerisation (about 70°C), but Taq DNA polymerase begins to work at the annealing temperature so that the extended products are long enough to remain attached to the template as the temperature reaches 72°C.

Oligonucleotides are usually used at a concentration of about 1 μM, which is sufficient for at least 30 cycles. If too high a concentration is used priming may occur at ectopic sites causing "false priming". At low oligonucleotide concentrations the reaction is inefficient.

1.3.2 Buffers

Standard PCR buffers contain 50 mM KCl, 10 mM Tris.Cl (pH 8.3 at room temp.) and 1.5 mM $MgCl_2$. Gelatin or bovine serum albumin (100 μg/ml) is also included to help stabilise the enzyme. The total reaction volume is 50-100 μl. At 72°C the pH drops by 1 unit to about 7.2. The presence of a divalent cation is critical for polymerase activity, and magnesium is better than either manganese or calcium (Chien *et al*, 1976). DNA templates should be stored in 10 mM Tris.Cl (pH 7.6) with low concentrations of EDTA (0.1 mM, pH 8.0), to prevent chelation of magnesium in the PCR reaction.

The concentration of magnesium may have to be titrated for different sets of primers/templates.

1.3.3 Taq DNA polymerase

Two forms of the enzyme are available: the native enzyme purified from Thermus aquaticus (a thermophilic, eubacterial micro-organism capable of growth at 70-75°C, first isolated from a hot spring in Yellowstone National Park - Brock and Freeze, 1969) and a genetically engineered form synthesised in E.Coli (AmpliTaq™). Both forms carry a 5' → 3' polymerisation-dependant exonuclease activity but they lack 3' → 5' exonuclease action. For nucleotide incorporation the enzyme works best at 75-80°C, depending on the target sequence, with nucleotides added to the free hydroxyl group at the 3' end of single stranded DNA at about 150/second/enzyme molecule (Gelfand and White, 1990). The polymerase activity is reduced by a factor of 2 at 60°C and a factor of 10 at 37°C. It is, however necessary to use it at sub-optimal temperatures to prevent dissociation of primer from template. The great advantage of this enzyme is its heat stability allowing the repeated denaturation steps in the PCR without any great loss of activity. At 95°C the T 1/2 is 40 minutes, compared to 5 minutes at 97.5°C and 130 minutes at 92.5°C. Approximately 2 units of the enzyme are required for each reaction. Higher concentrations may cause amplification of non-target sequences.

1.3.4 Deoxyribonucleoside triphosphates (dNTPs)

dNTPs are used at saturating concentrations of 200 μM each. Higher concentrations promotes misincorporation by the polymerase.

1.3.5 Target sequences

DNA can be used in either single or double stranded forms. The DNA is better amplified in linear form than circular. The concentration of target DNA is not easy to precisely control.

1.3.6 Cycling temperatures

An initial denaturation step of 95°C for 5-10 minutes can be used. After this the Taq DNA polymerase is added and a series of cycles performed:

Primer annealing - 2 minutes at 55°C
Primer extension - 3 minutes at 72°C
Denaturation - 1 minute at 94°C

There is usually a final 10 minute polymerisation step to ensure that all amplified DNA is double stranded, which is followed by a "soak" file at 4-15°C for an indefinite period.

The lower the primer annealing temperature the more efficient the amplification but the less specific the annealing. The optimum temperature should be determined for each set of primers.

Also the primer extension time may have to be lengthened if very long sequences (> 2Kb) are being amplified.

Denaturation time and/or temperature may have to be increased for GC rich targets, but if it is increased too long there will be unnecessary loss of enzyme activity.

The number of cycles is usually 25-30 (giving about 10^6 fold amplification). If many more cycles are to be used it is better to take a small fraction of this reaction and add it to fresh PCR buffer.

The PCR reaches an amplification plateau when the number of copies of the desired fragment stops accumulating exponentially. A number of factors contribute to this: exhaustion of primer or dNTP; inactivation of polymerase or dNTP; substrate excess (too much for the amount of Taq polymerase in the allotted extension time); competition by non-specific products (unwanted DNA competing for the polymerase); and product reassociation (at high concentrations single stranded fragments may reanneal before primer annealing).

1.4 Reverse transcription reaction

The reverse transcription reaction is carried out in a similar buffer but with higher magnesium concentration (15 mM $MgCl_2$), placental RNase inhibitor (1 unit/μl) and either moloney murine leukaemia virus (MoMuLV) reverse transcriptase or avian myeloblastosis virus (AMV) reverse transcriptase. AMV differs from the MoMuLV enzyme by: possessing powerful RNase H activity (degradation of RNA in RNA:DNA hybrids), restricting the length of cDNA sequences; working better at 42°C than 37°C, the preferred temperature of MoMuLV reverse transcriptase; and by working more efficiently at pH 8.3 compared to pH 7.6 for MoMuLV reverse transcriptase. This pH is very critical and should be measured at the correct incubation temperature. Both enzymes lack 3'→ 5' exonuclease activity and are therefore prone to error, especially at high concentrations of magnesium or dNTPs. Sometimes actinomycin D is added to the reaction to a final concentration of 50 μ g/ml to inhibit self-primed double stranded synthesis.

A number of priming methods can be used, either random hexamers, oligo(dT) or the anti-mRNA primer.

The reverse transcription reaction is incubated at 37-42°C for 30-60 minutes, before being denatured and adding to the PCR reaction.

2. RNA EXTRACTION AND PURIFICATION

2.1 Controlling RNase activity

A number of precautions must be taken prior to extraction of RNA to prevent contamination of the sample by ribonucleases (Sambrook *et al*, 1989). Sterile disposable plasticware is essentially RNase free, however, general laboratory glassware and plasticware can easily become contaminated. A number of methods can be applied to eliminate this risk:

(i) Glassware can be baked at 180°C for 8 hours.
(ii) Plasticware can be rinsed with chloroform.
(iii) Containers can be filled with diethylpyrocarbonate (DEPC) in a 0.1% solution in water, which is a strong inhibitor of RNases (Fedorcsak and Ehrenberg, 1966). After standing for 2 hours at 37°C it can be rinsed and autoclaved for 15 minutes at 15lb/sq.in. on the liquid cycle, to remove traces of DEPC which is a suspected carcinogen and can carboxymethylate purine residues in RNA.
(iv) Soaking in a 3% solution of H_2O_2 for 10 minutes followed by rinsing in DEPC treated water(see appendix).

It is useful to set aside designated laboratory glassware which is RNase free for use in these procedures (Sambrook *et al*, 1989). Gloves should be worn to prevent contamination of solutions or equipment with RNAses from the investigator's hands and should be changed frequently.

Solutions should be treated with 0.1% DEPC overnight and autoclaved before use, however, DEPC also reacts with amines so cannot be added to buffers such as Tris.

There are also specific RNase inhibitors including a protein derived from human placenta that binds RNases making them enzymatically inactive (Blackburn *et al*, 1977).

Guanidinium HCl and guanidinium thiocyanate have the advantage of being strong denaturing agents, so they readily dissociate nucleic acids from proteins and inactivate RNAses so are particularly useful in RNA extraction methods (Cox, 1968).

2.2 Extraction methods

Extraction of RNA from formalin-fixed tissues requires additional procedures to overcome the effects of cross linking, usually involving protease digestion.

Previous methods for RNA extraction from fixed tissues have been published (Ben-Ezra *et al*, 1991; Weizacker *et al*, 1991; Eldadah *et al*, 1991; Jackson *et al*, 1990). Most of these methods involve dewaxing in xylene, followed by washing in ethanol and proteinase K/SDS digestion, and a final phenol-chloroform-isoamylalcohol or a similar type of extraction.

One study (Jackson *et al*, 1990), which concentrated mainly on DNA extraction methods, compared proteinase K incubation, SDS incubation and boiling. Best results were obtained with proteinase K, and a 5 day digest produced substantially higher yields of DNA than shorter digests. Similar yields were obtained if the section was not dewaxed. For fresh tissue a 1 hour proteinase K digest was optimum. It was also noted that using only 10% of the total extract gave a brighter band than using the entire amount. Good quantities of RNA were also produced by this method.

Another method (Weizacker *et al*, 1991) looking specifically at RNA extraction used a similar method of digestion to that described above followed by phenol extraction and precipitation in 60% isopropanol in the presence of sodium acetate (pH 5.3) and glycogen. The extract was then subject to reverse transcription and PCR; and using 1-2 mg of liver tissue it was possible to amplify albumin mRNA. A similar method for extraction of RNA from fresh tissue has also been widely used (Chomczynski and Sacchi, 1987).

3. SOUTHERN BLOTTING

3.1 Agarose gel electrophoresis

Electrophoresis through a gel is the standard method to separate and identify DNA. The location of the DNA can be determined by staining with low concentrations of a fluorescent intercalating dye - ethidium bromide. Bands containing as little as 1-10 ng of DNA can be detected by direct examination with ultraviolet light (Sharp *et al*, 1973).

Gels can be made of either polyacrylamide or agarose. Polyacrylamide has the advantage of giving higher resolution and being able to separate fragments that differ in size by only one base pair. They have the disadvantage of being more expensive and difficult to prepare and involve the use of toxic substances. For most purposes agarose gels are adequate.

Agarose is a linear polymer of D-galactose-3,6-anhydro L- galactose, derived from seaweed. Gels are prepared by melting agarose in a suitable buffer until a clear solution is obtained and this is then poured into a mould. Once an electric current is applied across the hardened gel, DNA migrates towards the anode at neutral pH. The rate of migration is determined by a number of factors (Sambrook *et al*, 1989):

(i) Size of the DNA fragments - DNA becomes orientated in an end-on position and migration through the gel matrix is inversely proportional to the $\log_{10}$ of the number of base pairs, because of the frictional drag (Helling *et al*, 1974).
(ii) Agarose concentration - there is a linear relationship between the concentration of agarose and speed of mobility of a given DNA fragment. Different agaroses from different manufacturers also differ in their properties.
(iii) Conformation of the DNA - circular and linear DNA of the same molecular weights migrate at different speeds depending on the conditions of electrophoresis (Thorne, 1966). Ethidium bromide in the buffer can further alter this by uncoiling DNA and thereby reducing it's mobility.
(iv) Voltage - the higher the voltage the faster the migration, however, as voltage increases mobility of high molecular weight DNA increases differentially. To obtain maximum resolution of fragments greater than 2 Kb in size voltages of no more than 5V/cm are used.
(v) Direction of electric field - changing the direction of the electric field forces the DNA fragments to re-align in a new direction, this takes large molecules longer, so

can be used as a method of separating DNA fragments of 10,000 Kb (pulsed field electrophoresis).

(vi) Base composition and temperature - these factors do not significantly alter mobility with agarose gels (Thomas and Davis, 1975), in contrast to polyacrylamide gels.

(vii) Buffer - the higher the ionic strength the better the conduction, so the faster the mobility. If the concentration is too high there may be a lot of heat generated and the gel can melt and DNA denature. The most commonly used buffer is TAE, but it's buffering capacity is less than the slightly more expensive TPE and TBE, so if prolonged electrophoresis is carried out it may be necessary to recirculate between the two reservoirs.

3.1.1 Preparation of the gel

1. The edges of a plastic gel cast are sealed with autoclave tape and placed horizontal on the bench.
2. The correct amount of TAE is measured (volume depends on size of gel) and agarose is weighed out.
3. Agarose is added to the TAE and dissolved by heating (this may be performed in a microwave oven).
4. Once the solution is clear it can be poured into a gel cast (containing the comb) and left to set. This takes 1 hour at 4°C. The autoclave tape can then be removed and the gel is ready for use.

3.1.2 Electrophoresis

1. The DNA is mixed with a loading buffer (see appendix) and heated to 65°C for 10 minutes prior to use to ensure "sticky" fragments have separated.
2. The gel is placed into an electrophoresis tank and covered with TAE, to reach about 1 mm above the gel.
3. The comb is carefully removed from the gel.
4. DNA samples are pipetted into the submerged wells.
5. An electric current is applied at 1-5 V/cm (e.g. 40V for a minigel, 60V for a midigel and 100V for a large gel).
6. The gel is run until the dye reaches 2/3 of the way down (approximately 4-5 hours).
7. The gel is then removed and stained in ethidium bromide (1 μg/ml) for 1 hour.

8. The stained gel is rinsed in tap water and photographed in ultraviolet light. The emitted light is at about 366nm and is best photographed on Polaroid type 57 cor 667 (ASA 3000) film with a Wratten 22A filter.

N B. ethidium bromide can be added to the gel during preparation so that it can be monitored during electrophoresis, however, it reduces the speed of migration of linear double-stranded DNA by about 15% and may produce less sharp bands.

3.1.3 Size marker

A number of different size markers can be used to assess the migration and size of bands. For this study the DNA size marker used is ϕX 174 DNA (Hae III digest), which produces bands of the following base pair sizes: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72. A small aliquot of the DNA is mixed with loading buffer and loaded into a well before electrophoresis.

3.2 Southern blotting

Once the DNA has been run in a gel it must be transferred on to a nylon membrane or nitrocellulose filter before it can be identified by hybridisation with a labelled probe. This process of transfer is known as Southern blotting (Southern, 1975). As well as identifying the DNA fragment the technique adds further sensitivity, allowing as little as 0.1 pg of DNA to be identified.

DNA can be transferred on to a solid support by the aid of capillary action after a denaturation step e.g. by immersion in alkali. The gel is then placed on to Whatman 3MM paper immersed in a transfer buffer. On top is placed the nitrocellulose or nylon with a further 3MM paper and a stack of paper towels weighed down with a 500g weight. The speed of transfer depends on the size of the fragments and concentration of agarose in the gel. With transfer of large fragments (e.g. >15 Kb) a depurination/hydrolysis step can be used to aid transfer.

DNA can also be transferred with the aid of electrophoresis, which can be technically difficult or be the aid of a vacuum transfer device (allowing more rapid transfer).

Initial transfers were on to nitrocellulose filters, however the disadvantage of these are that DNA is only attached by hydrophobic bonds, so may diffuse, and the filters

become brittle and therefore do not allow repeated cycles of washing and hybridisation. Nylon membranes do not have these disadvantages and can be used repeatedly and transfer may be carried out at low ionic strength allowing electrophoretic transfer, which is of particular use with polyacrylamide gels. The main disadvantage of nylon is the increased background, needing several blocking agents. DNA is fixed to the nylon by either vacuum drying or UV light (254 nm) following transfer.

3.4 Probing to the filter

The filter is incubated with a prehybridisation solution containing salmon sperm DNA, NaCl, SDS, formamide and dextran sulphate at 37°C for 1 hour. Then ^{32}P labelled probe is added and hybridised overnight at 37°C. Post-hybridisation washes consist of SSC/formamide/Triton X at 37°C. The filter is then blot-dried, wrapped in saran and exposed to X-ray film at -70°C with an image intensifying screen for 4-120 hours, in order to obtain an autoradiographic image.

4. PRIMER DESIGN

4.1 General principles

Design of suitable primers is critical to successful PCR. A few simple rules should be followed (Sambrook *et al*, 1989):

(i) Select primers with a random base distribution and GC content similar to that of the fragment being amplified (ideally 50-60% GC rich). Avoid runs of polypurines or polypyrimidines.
(ii) Sequences with significant secondary structure should be avoided, particularly at the 3' end.
(iii)Check that the primers are not complimentary. Primers with overlap at the 3' end will produce "primer dimers". These are an amplification artefact, more commonly observed when template concentration is low and it is a double stranded fragment with a similar length to the sum of the primers.

A primer length of 18-24 nucleotides is adequate to achieve specificity.

4.2 Glyceraldehyde-3-phosphate dehydrogenase primers

Primers for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA were chosen as a positive control because of it's relatively high expression in skeletal muscle (Piechaczyk *et al*, 1984). This enzyme has an important function in glycolysis and appears highly conserved between species (Fort *et al*, 1985). However, like many important cellular genes there are numerous pseudogenes (Tso *et al*, 1985) which are problematic for RNA PCR because pseudogenes have very similar sequences to mRNA (Galland *et al*, 1990).

Control primers to demonstrate G3PDH were designed around introns to produce a fragment of 2086 base pairs from the DNA and 234 base pairs from mRNA. One of the primers also overlapped an intron/exon boundary further reducing the chances of amplification of nuclear DNA.

Primers were chosen be 21 nucleotides in length and to have a GC content of 51%. The primer sequences were complimentary to both human and rat mRNA sequences. An internal probe of 30 nucleotides was selected with a similar GC content (sequence data is given in Table 12).

4.3 Coxsackie virus primers

Recently a number of PCR methods have been published for the detection of picornaviruses from clinical specimens (Hyypia *et al*, 1989; Olive *et al*, 1990; Rotbart *et al*, 1990; Chapman *et al*, 1990; Jin *et al*, 1990; Weiss *et al*, 1991; Gow *et al*, 1991; Leff *et al*, 1992). Most of these publications have used primers derived from the highly conserved 5' UTR which detect most picornaviruses tested (with the exception of Echo virus 22). However, one study used the P2 region of coxsackie B3 (Weiss *et al*, 1991) and appears specific to this serotype.

For the purpose of this study primers were designed to detect all strains of coxsackie B virus and a relatively small fragment was amplified to minimise the problem of fragmentation of nucleic acids which can occur in formalin-fixed paraffin-embedded tissue. Of the primers already used in the above publications, those used by Rotbart (Rotbart *et al*, 1990), which were very similar to those used by Jin (Jin *et al*, 1990), appeared most appropriate. The fragment amplified was 150 -180 base pairs in length, and because of the high degree of conservation in this region it would also be possible to design internal primers to the amplified fragment for "nested" PCR. The sequences were modified slightly from those published so that both primers were 21 nucleotides in length with an identical GC content (47.6%); this balancing of the primers is important for them to work under the same conditions and to work equally well, avoiding asymmetrical amplification of one strand in preference to the other. Also both primers were moved to end on either a G or C base to ensure tight annealing at the 3' end. The modified primers produced a fragment of 145 base pairs in length. One of the probes used for *in situ* hybridisation (CXB 4) was used as an internal probe to confirm the identity of the amplified fragment. Sequence details are given in Table 12.

4.4 "Nested" primers

In order to further increase both sensitivity and specificity primers were designed to the amplified fragment from the first set of coxsackie primers. Sequences were identified in this region with a GC content of about 50%. One "nested" primer (coxsackie primer 3) overlapped coxsackie primer 1 as no other sequences fulfilled these criteria. Primer sequences are given in the Table 12:

Table 12 - Primer and internal probe sequences

Primer/probe	Sequence	Position
Coxsackie primer 1 (upstream)	CTG.AAT.GCG.GCT.AAT.CCT.AAC	457-477*
Coxsackie primer 2 (downstream)	CAA.TTG.TCA.CCA.TAA.GCA.GCC	602-582*
Coxsackie primer 3 ("nested")	CTA.ATC.CTA.ACT.GCG.GAG.CAC	467-487*
Coxsackie primer 4 ("nested")	ATG.AAA.CAC.GGA.CAC.CCA.AAG	567-547*
Coxsackie probe (internal probe)	TCG.GTT.CCG.CTG.CAG.AGT.TGC.CCG.TTA.CGA	542-513*
G3PDH primer 1 (upstream)	AGG.TGA.AGG.TCG.GAG.TCA.ACG	1460-1480**
G3PDH primer 2 (downstream)	GCT.CCT.GGA.AGA.TGG.TGA.TGG	3543-3542 & 3412-3394**
G3PDH probe (internal probe)	ACC.ATG.TAG.TTG.AGG.TCA.ATG.AAG.GGG.TC	3307-3306& 3413-3190**

*sequence from coxsackie B3 virus (Klump *et al*, 1990)
**sequence from G3PDH gene (Ercolani *et al*, 1988)

5. DEVELOPMENT OF PROTOCOLS

5.1 RNA extraction methods

A number of extraction methods for paraffin embedded sections (using the mice infected with coxsackie virus as a control) were tried, including:

(i) simple boiling of tissue sections.
(ii) dewaxing sections followed by digestion with proteinase K with SDS.
(iii) dewaxing sections followed by digestion with proteinase K without SDS.

These were followed by purification with phenol-chloroform-isoamylalcohol extraction, and a final ethanol precipitation step.

Simple boiling and digestion using proteinase K alone, did not produce any bands after reverse transcription and PCR, even when using sections containing large amounts of coxsackie virus (by ISH). However, if SDS was added to the digest good bands were produced. The SDS was therefore essential to the procedure. The proteinase K/SDS was incubated for periods of 1 hour, 4 hours, overnight (12 hours) or 5 days; of these overnight digestion produced the strongest bands after reverse transcription and PCR.

The purification with phenol-chloroform-isoamylalcohol followed by ethanol precipitation [add equal volume of phenol-chloroform-isoamylalcohol (25:24:1) centrifuge and extract upper aqueous layer; add equal volume chloroform-isoamylalcohol (24:1) centrifuge and extract aqueous layer; add 1/10 volume 1M NaCl and 3 x volume cold absolute ethanol and precipitate at - 20^{o}C for 30-60 minutes] was adequate if the section contained large amounts of virus but not if only 1-5 cells were infected with virus, as judged by ISH.

Therefore another extraction method was tried. One method now widely used is the acid-phenol-guanidinium-isothiocyanate extraction method (Chomczynski and Sacchi, 1987), however, there have been no publications using this method on fixed tissue. The extraction relies largely on the ability of guanidinium isothiocyanate to disrupt hydrogen bonds, but this does not break the covalent bonds formed during fixation with formaldehyde, therefore the extraction process was adapted to follow on from proteinase K digestion (see appendix 2).

This latter method gave much improved results and was adopted for the rest of the study. The extraction was applied directly to frozen tissue without the need for digestion.

5.2 Cutting sections and optimisation of the amount of tissue used

Paraffin embedded tissue blocks were cut on a microtome using disposable blade and disposable plastic forceps to pick up the sections and place them into a sterile 1 ml eppendorf tube. The blade and it's clamp were washed thoroughly with xylene between each case and new plastic forceps used for each case in order to reduce the chances of cross contamination of tissue.

To optimise the amount of tissue for amplification, a comparison of using either 100%, 50%, 10% or 1% of either 4 x 10 μm, 2 x 10 μm or 1 x 10 μm sections (containing small amounts of coxsackie virus) was carried out. Initially 4 μm sections were used, but I found that during the extraction procedure the sections often fragmented, with subsequent loss of tissue during xylene/ethanol washes. This did not occur with 10 μm sections.

The strongest bands were achieved using either 10% of the extract from 4 x 10 μm sections or 10% of the extract from 1 x10 μm section. This experiment was also repeated using the G3PDH primers on human tissue (post-mortem heart, spinal cord and a skeletal muscle biopsy) with the same results (see Figure 38). Tissue appears to contain inhibitors of PCR (Fleming and An, 1991) which are not eliminated by the above extraction methods, and this probably accounts for the weaker bands with larger amounts of tissue extract.

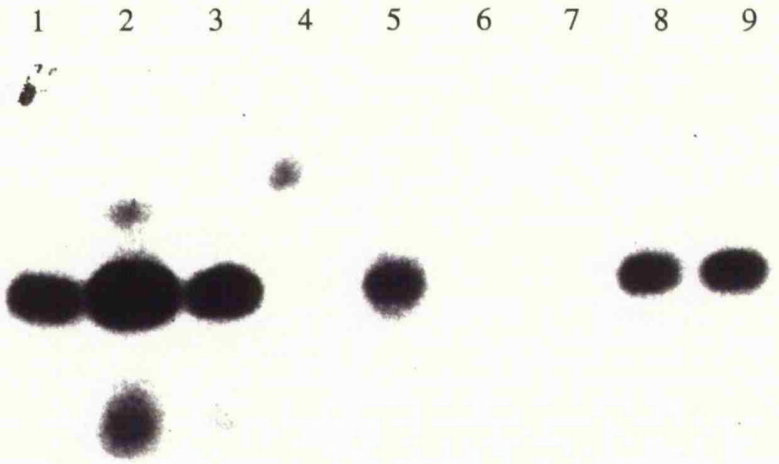


Figure 38 - Southern blot of gel probed with ^{32}P labelled internal G3PDH probe following amplification with G3PDH primers using different amounts of template.

Lane 1 - 10% extract from 10 x 10 μm paraffin sections of normal skeletal muscle.
Lane 2 - 10% extract from 4 x 10 μm paraffin sections of normal skeletal muscle.
Lane 3 - 10% extract from 1 x 10 μm paraffin sections of normal skeletal muscle.
Lane 4 - 10% extract from 10 x 10 μm paraffin sections of normal post-mortem spinal cord.
Lane 5 - 10% extract from 4 x 10 μm paraffin sections of normal post-mortem spinal cord.
Lane 6 - 10% extract from 1 x 10 μm paraffin sections of normal post-mortem spinal cord.
Lane 7 - 10% extract from 10 x 10 μm paraffin sections of normal post-mortem myocardium.
Lane 8 - 10% extract from 4 x 10 μm paraffin sections of normal post-mortem myocardium.
Lane 9 - 10% extract from 1 x 10 μm paraffin sections of normal post-mortem myocardium.

5.3 RNA denaturation

Initially all cases i.e. those with and those without virus produced positive bands of about 400 base pairs in size, when using the coxsackie primers, but not with the G3PDH primers. This band was only produced if the reverse transcriptase was added, proving it to be originating from RNA. However, after heating the RNA extract to 94°C for 10 minutes, followed by cooling rapidly on ice, prior to reverse transcription the band disappeared (see Figure 39). This extra band is therefore probably related to secondary structure of a host RNA, which must be in high copy number judging by the strength and consistency of this band. One possibility is that ribosomal RNA may share complimentary sequences to the conserved regions of the

5'UTR of the virus from where the primer are derived (this portion of the virus genome is strongly implicated in ribosomal interaction - see introduction). When the ribosomal RNA has it's secondary structure removed the regions may become fragmented.

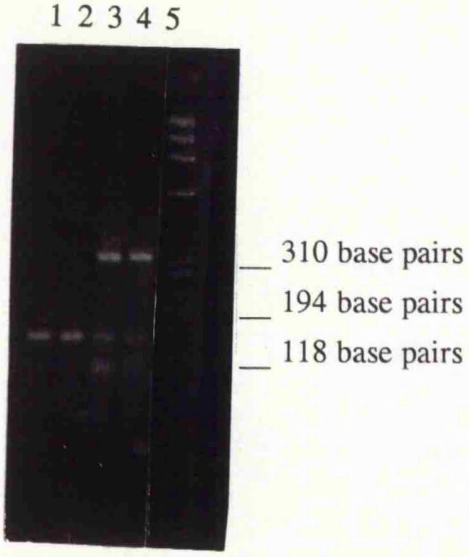


Figure 39 - Agarose gel stained with ethidium bromide following amplification of extract from paraffin sections of coxsackie B virus infected mice using the coxsackie primers 1 and 2, with and without heating of extract, and comparing 37°C with 42°C for reverse transcription.

Lane 1 - Extract heated to 94°C prior to reverse transcription at 37°C with AMV enzyme.
Lane 2 - Extract heated to 94°C prior to reverse transcription at 42°C with AMV enzyme.
Lane 3 - Extract not heated prior to reverse transcription at 37°C with AMV enzyme.
Lane 4 - Extract not heated prior to reverse transcription at 42°C with AMV enzyme.
Lane 5 - Size marker ϕX 174 DNA (Hae III digest).

As well as removing this "artefact" band, heating the RNA also greatly increased the intensity of the 147 base pair virus band.

5.4 Optimisation of buffer for reverse transcription

To obtain the best reverse transcription reaction, for subsequent PCR, different buffers were compared:

PCR buffer	RT buffer	Commercial RT buffer(BRL)
45mM Tris, pH 8.8	50mM Tris, pH 8.3	50mM Tris, pH 8.3
11mM $(NH_4)_2SO_4$	75mM KCl	40mM KCl
4.5mM $MgCl_2$	3mM $MgCl_2$	6mM $MgCl_2$
50µM dNTPs	500µM dNTPs	500µM dNTPs
110µg/ml BSA	100µg/ml BSA	100µg/ml BSA
	10mM DTT	10mM DTT
		50µg/ml Actinomycin D

Reverse transcription was carried out in a volume of 50 µl, with either 5U AMV (Boehringer Mannheim) or 200U MoMuLV (Gibco BRL) reverse transcriptase at 37°C for 1 hour. The downstream coxsackie primer (Coxsackie primer 2) was used to initiate reverse transcription. 1 or 10 µl of the final reaction volume was used for subsequent PCR with the coxsackie primers.

RNA extracted from coxsackie B3 infected mice was used for this experiment. The strongest bands were obtained with the PCR buffer.

No significant difference was noticed between the two enzymes, and both enzymes worked well at either 37°C or 42°C. AMV was used, 2.5 units were as good as 5 units in a final reaction volume of 20 µl, but a reduction in band intensity was noticed at lower enzyme concentrations.

Adding actinomycin D or DTT to the PCR buffer prior to reverse transcription had a detrimental effect.

Varying the pH from 8.0 - 8.8 and $[Mg^{++}]$ from 0 - 5 mM was carried out with an optimum pH of 8.4 and $[Mg^{++}]$ of 2mM for reverse transcription. Increasing the concentration of dNTPs also produced a slight benefit.

A final buffer for reverse transcription, based on the original PCR buffer was used, so that a 20 µl reaction was carried out and the PCR carried out in the same tube, thereby minimising handling and allowing all the cDNA produced to be used for amplification.

5.5 Final reverse transcription reaction

2 µl of extracted RNA (heated to 94°C for 10 minutes then chilled on ice) is added to 18µl of a reaction buffer containing:

45mM Tris, pH 8.4
11mM $(NH_4)_2SO_4$
2mM $MgCl_2$
250µM dNTPs
110 µg/ml BSA
50 pmoles of the downstream primer
4 U placental RNase inhibitor (Boehringer Mannheim)
2.5 U AMV (Boehringer Mannheim)

The reaction is incubated for 1 hour at 37°C.

5.6 Primers for first strand synthesis

The downstream primer (50 pmoles) was compared with a similar amount of random hexamers in the reverse transcription reaction. Both methods produced bands after PCR, but the specific primer was slightly better.

5.7 PCR conditions

Although no previous RT-PCR had been carried out within the department much developmental work had already been carried out in order to optimise conditions for PCR (using primers of the same GC content and length as mine).

The PCR buffer used was that used by Professor A. Jeffries (University of Leicester), and differs from conventional PCR buffers by the use of ammonium sulphate instead of potassium chloride. This buffer was found to produce much more reliable results than previous buffers, so has been adopted.

PCR was carried out in a reaction volume of 50 µl, instead of the 100 µl used by some, because only this amount could be run out in a gel so a larger volume results in extra cost (double the amount of enzyme, buffer and primers).

5.7.1 Enzyme

The optimum amount of DNA Taq polymerase was 0.5 U in the 50 µl, any higher amounts produced extra bands due to false priming. However, stronger bands were produced if a further 0.5 U of Taq were added after the first 20 cycles, presumably due to of denaturation of enzyme and/or increase in the amount of target. It was also found that adding the Taq after the initial 10 minute denaturation step produced much stronger bands than adding it at the beginning.

5.7.2 Annealing temperature

Annealing temperatures of 55-65°C were compared. An optimum of 62°C was found with all sets of primers.

5.7.3 Magnesium concentration

Concentrations of 0-10mM $MgCl_2$ were tried, 4-5mM being optimum for the PCR, but 2mM was better for reverse transcription.

5.7.4 Number of cycles

Using sections with only a small amount of virus it was found that 40 cycles were needed to produced consistent bands, compared with 30-35 if large amount of virus was present.

5.7.5 Cycling temperatures

After the reverse transcription, carried out at 37-42°C the mixture was denatured at 98°C for 10 minutes followed by an annealing step at 62°C for 10 minutes at which time the Taq was added, followed by 2 minutes of extension at 72°C.

After this a thermocycle file is carried out consisting of:

Denaturation at 96°C for 30 seconds
Primer annealing at 62°C for 30 seconds
Primer extension at 72°C for 1 minute, 30 seconds

After 40 cycles there is a final extension of 10 minutes at 72°C before a soak file at 4°C.

Initially longer denaturation and annealing times were used (about 1.5 minutes), but for the short target sequences used in this study (all less than 300 base pairs) the above times produced identical results.

6. PCR AMPLIFICATION OF DIFFERENT VIRUSES

PCR of extracted RNA from a range of tissues containing different virus strains was carried out to ensure that the coxsackie virus primers amplified all strains of coxsackie and did not cross react with unrelated viruses.

Investigation of tissue sections from mice infected with the different coxsackie strains and uninfected mice was carried out. In addition tonsillar tissue shown to contain Epstein-Barr virus by *in situ* hybridisation to viral RNA; acutely diseased liver from a case of hepatitis B virus infection (demonstrated by immunocytochemistry); brain from a case of encephalitis due to herpes simplex virus type I (demonstrated by immunocytochemistry) and small intestine from a case of AIDS related cytomegalovirus infection (demonstrated by *in situ* hybridisation to viral DNA) were investigated. Also spinal cord tissue from monkeys infected with each of the three strains of polio virus were kindly donated by Dr P.D.Minor at the National Institute for Biological Standards and Control, Potters Bar, Hertfordshire.

RNA was extracted (as described above) from paraffin embedded tissue sections in all cases and the results of reverse transcription followed by 40 cycles of the PCR are shown in Figure 40. All of the coxsackie strains were detected and weak bands were also seen with polio viruses (types 1-3). No bands were seen with any of the other unrelated viruses. However, on Southern blotting of the reaction product and probing with ^{32}P labelled internal probe all the positive bands were detected with the exception of coxsackie A21 virus and polio virus type 3 (Figure 41).

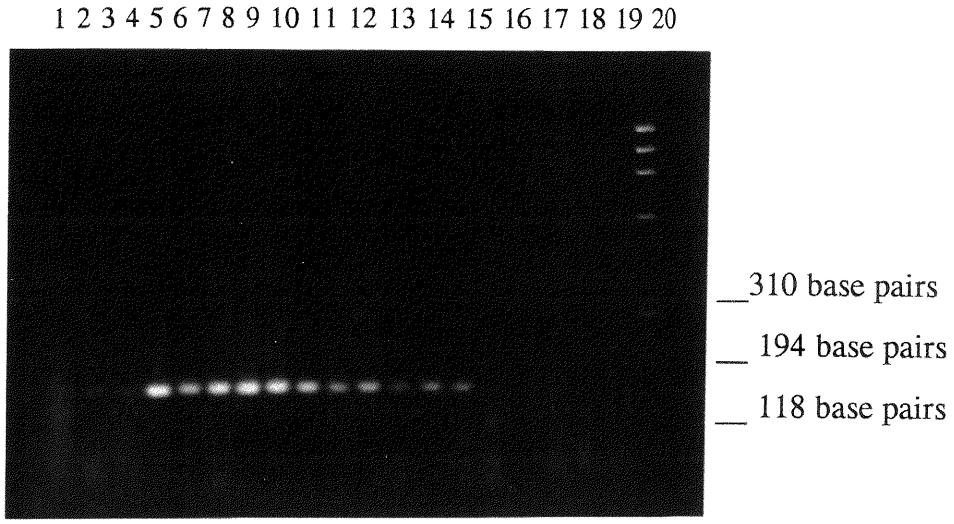


Figure 40 - Agarose gel stained with ethidium bromide following reverse transcription-PCR using the coxsackie virus primers 1 and 2 on extracts from paraffin embedded tissue sections on a range of virus strains.

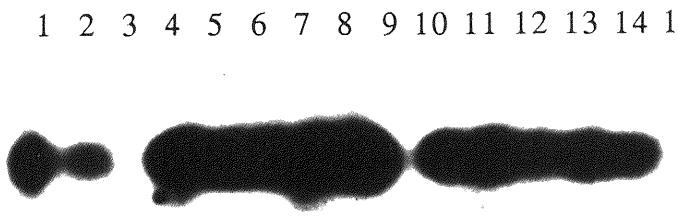


Figure 41 - Southern blot of gel in Figure 40, hybridised with ^{32}P labelled internal coxsackie virus probe.

Lane 1 - Polio virus type 1
Lane 2 - Polio virus type 2
Lane 3 - Polio virus type 3
Lane 4 - Coxsackie virus A5
Lane 5 - Coxsackie virus A7
Lane 6 - Coxsackie virus A8
Lane 7 - Coxsackie virus A10
Lane 8 - Coxsackie virus A16
Lane 9 - Coxsackie virus A21
Lane 10 - Coxsackie virus B1
Lane 11 - Coxsackie virus B2
Lane 12 - Coxsackie virus B3
Lane 13 - Coxsackie virus B4
Lane 14 - Coxsackie virus B5
Lane 15 - Uninfected mice
Lane 16 - Epstein-Barr infected tonsil
Lane 17 - Hepatitis B infected liver
Lane 18 - Herpes simplex type I infected brain
Lane 19 - Cytomegalovirus infected bowel
Lane 20 - ϕX 174 DNA (Hae III digest)

In addition it was shown that virus RNA could be detected using only 0.01% of the extracted RNA from a single 10 μm section of coxsackie A10 infected mouse (Figure 42).

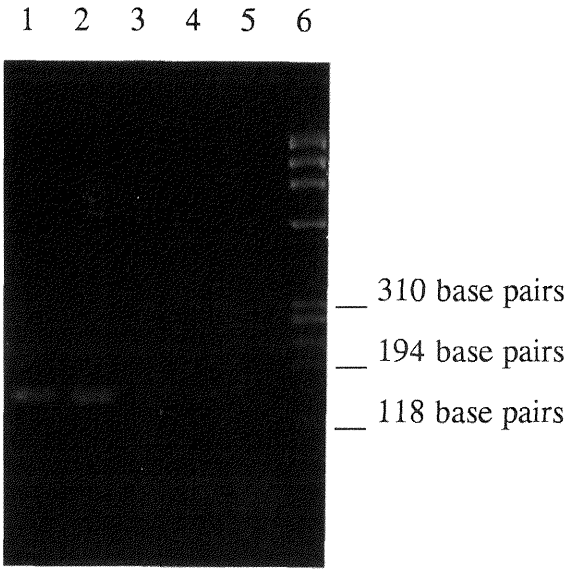


Figure 42 - Agarose gel stained with ethidium bromide of reverse transcription-PCR, with coxsackie primers 1 and 2, from different amounts of the extract of a single section of coxsackie A10 virus infected mouse tissue.

Lane 1 - 10% of tissue extract.
Lane 2 - 1% of tissue extract.
Lane 3 - 0.1% of tissue extract.
Lane 4 - 0.01% of tissue extract.
Lane 5 - No template.
Lane 6 - ϕX 174 DNA (Hae III digest).

The use of "nested" primers for the detection of virus RNA was also investigated. Using 1 μl of the PCR product from the first 40 cycles (using primers 1 and 2), the sample was subject to a further 20 cycles of the PCR with the internal coxsackie primers (3 and 4). This was shown to detect all strains of coxsackie and polio virus examined producing a 100 base pair fragment with an increased sensitivity to one round of 40 cycles. The extra sensitivity appeared to be approximately equivalent to the increased sensitivity produced by Southern blotting and probing with ^{32}P labelled oligonucleotides. However, "nested" PCR did have the disadvantage of detecting low levels of contamination (see later in section 8).

7. PCR AMPLIFICATION OF MYOCARDITIS CASES

The 10 cases of childhood myocarditis and 10 controls cases investigated by *in situ* hybridisation for the presence of coxsackie virus (see part 2, section 5) were studied by PCR for the presence of virus and with control primers for G3PDH. Details of the cases are given in Tables 11 and 12. RNA was extracted from single 10 μm sections of myocardium as described above and subjected to reverse transcription and the PCR in separate reaction tubes, with either G3PDH primers or coxsackie primers. After 40 cycles the reaction products were stained with ethidium bromide and are shown in the gel below (Figure 43). As can be seen the 2 cases positive by *in situ* hybridisation were also positive by PCR for the presence of virus. None of the remaining cases or controls had any evidence of virus by PCR. No bands were seen with the G3PDH primers in any of the cases. However, following southern blotting of the product and probing with ^{32}P labelled internal probes bands for G3PDH mRNA were seen in 8 cases and 8 controls, but no extra positive cases were seen with virus amplification.

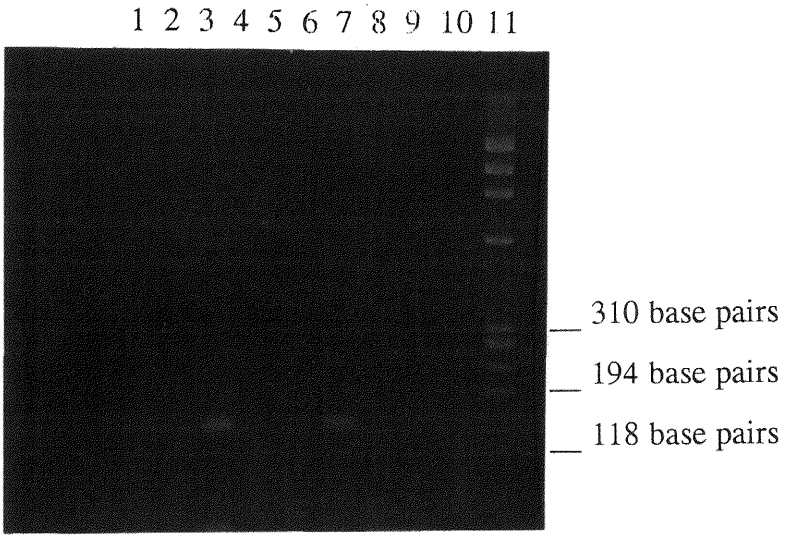


Figure 43 - Agarose gel stained with ethidium bromide showing the results of reverse transcription-PCR with coxsackie virus primers 1 and 2 on myocarditis cases.

Lanes 1-10 - myocarditis cases 1-10.
Lane 11 - ϕX 174 DNA (Hae III digest).

These results show that there is good correlation between the techniques of reverse transcription PCR and *in situ* hybridisation for the detection of coxsackie virus. The absence of virus in the cases of myocarditis negative by *in situ* hybridisation makes the possibility of small amounts of virus causing the myocardial inflammation and necrosis very unlikely. The aetiology and pathogenesis of myocardial damage in the negative cases remains unknown, but may be due to lytic infection of myocardial cells by unrelated viruses (Woodruff, 1980), or autoimmune mechanisms as suggested by others (Rose *et al*, 1988).

The lack of any visible bands with the G3PDH primers following reverse transcription-PCR and ethidium bromide staining of the gel, but presence in 80% of cases following probing to the Southern blots, demonstrates the increased sensitivity gained by probing with ^{32}P labelled oligonucleotides (also demonstrated in section 5.2). Also all of these cases were probed to by *in situ* hybridisation with a cocktail of 3 oligonucleotides to G3PDH (see section 2.2 for sequences), labelled with digoxigenin, and none was positive. This demonstrates that reverse transcription-PCR followed by Southern blotting is much more sensitive than *in situ* hybridisation.

8. PCR AMPLIFICATION OF CASES OF IIMD

The 26 cases of IIMD examined by *in situ* hybridisation were also used for analysis by the PCR for the presence of coxsackie virus RNA and G3PDH mRNA. The sections were cut as described previously, however some of the cases had blocks of muscle which were becoming very small (i.e. only 2-3mm across), so in these cases up to eight 10 μm sections were used instead of a single section.

For frozen blocks the sections were not dewaxed or digested overnight with proteinase k, but initially cut in the cryostat at -20°C and simply put directly into the 100 μl solution D mixture (step 6 in RNA extraction protocol - see appendix 2). However, this approach was technically difficult as it was not easy to transfer the section from the cryostat blade to inside an eppendorf tube without it dropping. Also it was not possible to clean the blade adequately between cases, risking cross contamination. Therefore the RNA was extracted from these cases by shaving off a thin section (approximately 20 μm thick) from each case with a sterile disposable scalpel blade, changing the blade between cases.

Initial results with paraffin embedded material from 19 cases of IIMD produced very variable results with the primers for G3PDH (Figure 44) with bands present in only 9 cases (1 4,6,7,19,21,22,24 and 25). All the cases were negative for coxsackie virus after 40 cycles with coxsackie primers 1 and 2 (Figure 45), but positive bands in 7 cases following "nested" PCR with coxsackie primers 3 and 4 (cases 2,4,9,13,20,21 and 22) (Figure 46).

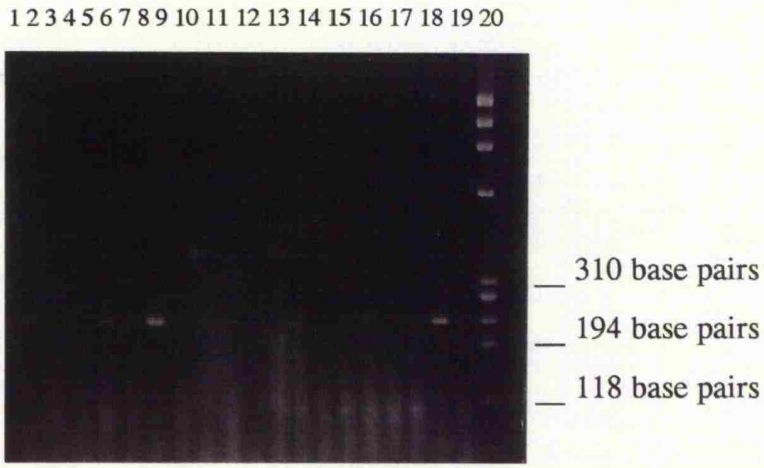


Figure 44 - Agarose gel stained with ethidium bromide showing results of reverse transcription-PCR using the G3PDH primers, of 19 IIMD cases (paraffin embedded material).

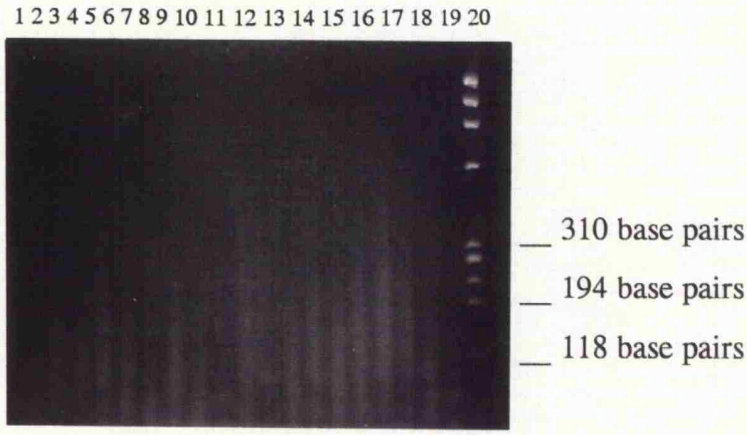


Figure 45 - Agarose gel stained with ethidium bromide showing initial results of reverse transcription-PCR using the coxsackie virus primers 1 and 2, of the same 19 IIMD cases.

Lane 1 - case 1
Lane 2 - case 2
Lane 3 - case 3
Lane 4 - case 4
Lane 5 - case 6
Lane 6 - case 7
Lane 7 - case 8
Lane 8 - case 9
Lane 9 - case 13
Lane 10 - case 15
Lane 11 - case 18
Lane 12 - case 19
Lane 13 - case 20
Lane 14 - case 21
Lane 15 - case 22
Lane 16 - case 23
Lane 17 - case 24
Lane 18 - case 25
Lane 19 - case 26
Lane 20 - ϕX 174 DNA (Hae III digest)

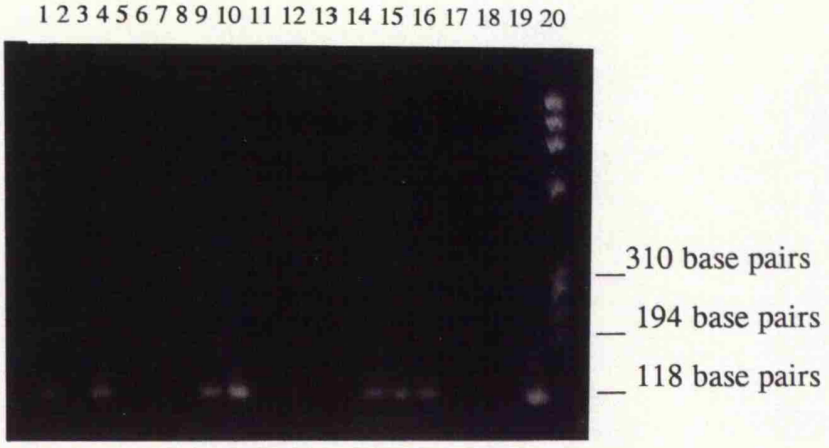


Figure 46 - Agarose gel stained with ethidium bromide showing results of "nested" PCR using the coxsackie primers 3 and 4, of the product shown in Figure 45.

Repetition of the "nested" coxsackie PCR amplification of these cases produced positive bands in more of the cases and virus bands were also produced if the reverse transcription step was omitted (unlike the bands with G3PDH). These bands were therefore due to contamination of samples by DNA.

The large number of positive bands suggested contamination of a solution. All solutions used for reverse transcription and the PCR, were changed, including those used for extraction of RNA from tissues. RNA was then extracted again from the cases of IIMD and PCR for coxsackie repeated. However, again after "nested", but not simple amplification, positive bands for virus were seen in 6 cases (2, 4, 7, 8, 22 and 23). These positive cases did not match with the previous positive cases and was still likely to be due to contamination. The only solutions not changed were the primer aliquots, so these were re-diluted from concentrated stocks. Also all the pipettes were cleaned thoroughly with detergent and sterile water, followed by 99% IMS.

Fresh RNA samples were again extracted from the muscle biopsies with repeat coxsackie virus RNA and G3PDH mRNA amplification. This time none of the cases were positive for virus, even after southern blotting and probing to the cases.

Amplification of frozen blocks of muscle produced much more consistent and stronger bands with the G3PDH primers indicating the superior RNA preservation/availability of frozen muscle compared to that of fixed muscle. The results of reverse transcription-PCR with G3PDH and coxsackie primers is shown in Figure 47, with the Southern blot in Figure 48.

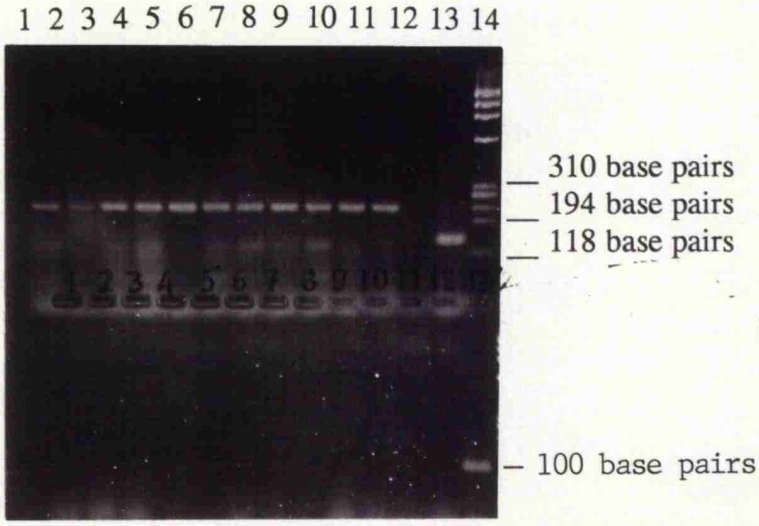


Figure 47 - Agarose gel stained with ethidium bromide following amplification of extracts from 11 frozen blocks from IIMD cases, with G3PDH and coxsackie primers. The first row contains the product from amplification using the G3PDH and coxsackie 1 and 2 primers (25 µl of product from each loaded into the same well for each case) with the "nested" PCR product from the same cases in the second row.

Lane 1 - case 5
Lane 2 - case 6
Lane 3 - case 10
Lane 4 - case 11
Lane 5 - case 12
Lane 6 - case 13
Lane 7 - case 14
Lane 8 - case 15
Lane 9 - case 16
Lane 10 - case 17
Lane 11 - case 18
Lane 12 - no template control
Lane 13 - coxsackie B2 virus infected tissue
Lane 14 - ϕX 174 DNA (Hae III digest)

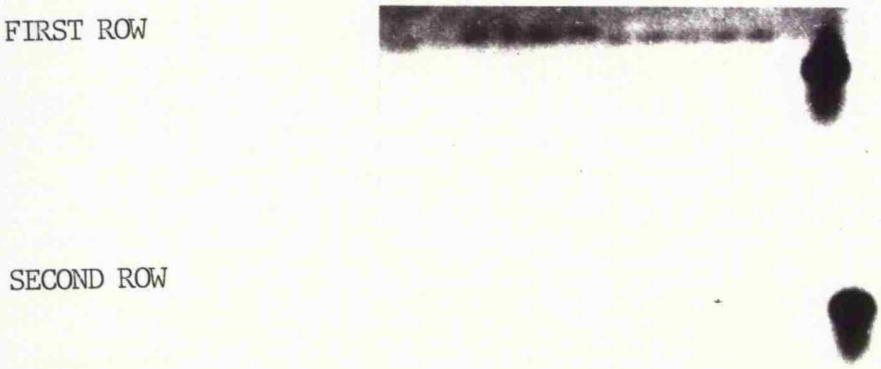


Figure 48 - Southern blot of the gel from Figure 47, after hybridisation with ^{32}P labelled internal probes to G3PDH and coxsackie virus PCR products.

These PCR results therefore do not demonstrate any evidence of virus persistence within the muscle of cases of IIMD, even after the more sensitive "nested" PCR and Southern blotting of the product.

PART 4

ULTRA-STRUCTURAL STUDY OF MUSCLE

1. Introduction

No systematic ultra-structural studies of cases of IIMD have been carried out, however, several claims for the presence of virus particles within muscle from cases have been based on ultra-structural appearances (see section 4.3.). As discussed this evidence is of doubtful significance. In order to further investigate the nature of "virus-like" particles within muscle I looked at all cases of IIMD where appropriate resin embedded tissue was available for the presence of such particles. These findings could then be compared to the results of *in situ* hybridisation and the PCR.

2. Methods

Blocks of approximately $1mm^3$ had been taken from muscle biopsy samples and fixed in 2.5% glutaraldehyde for 24-48 hours, and then post-fixed in 1% osmium tetroxide and 7.5% magnesium uranyl acetate. The samples were then processed to Spurr resin for transmission electron microscopy. Of the 17 LRI cases 14 had material available for ultra-structural examination.

Semi-thin sections were stained with toluidine blue and ultrathin sections with lead citrate. Semi-thin sections were cut from 6 resin blocks in each case and from these 3 were selected for ultrastructural examination, on the basis of muscle fibre necrosis, regeneration or inflammation being present.

Sections were briefly screened at low power (x1100) to find areas of interest ,such as inflammation and necrosis, then at higher power (x19000-2500). A minimum of 20 minutes per grid and 60 minutes per case was spent examining the muscle with the electron-microscope (Joel Tem-Scan 100) looking for various structures thought to be of possible viral origin (tubulo-reticular structures, filamentous structures and paracrystalline arrays). The presence or absence of inflammatory infiltrates and muscle fibre necrosis in the ultra-thin sections was also noted.

As a positive control skeletal muscle from one of the mice infected with coxsackie A21 was also processed for ultra-structural examination.

3. Results

Table 13 - Summary of ultra-structural findings

case	inflammation	muscle necrosis	tubulo-reticular inclusions	filamentous inclusions	prominent glycogen*
1**					
2**					
3	yes	yes	no	no	yes
4	yes	no	no	no	no
5	yes	yes	no	no	no
6**					
7	yes	yes	no	no	no
8	yes	yes	no	yes	no
9	yes	yes	no	no	yes
10	yes	yes	no	no	no
11	yes	yes	no	yes	no
12	no	no	no	no	no
13	yes	yes	no	no	no
14	yes	yes	yes	no	no
15	yes	no	no	no	yes
16	yes	yes	no	yes	no
17	yes	yes	no	yes	no

* no cases had paracrystalline arrays , however in some glycogen was very prominent.

** no tissue was available for ultra-structural examination in these cases.

Nearly all of these LRI cases had areas of active disease occurring in the tissue examined at EM, yet none showed any paracrystalline arrays, although the prominent amounts of glycogen in 3 cases did resemble picornavirus particles in size and shape (Figure 49). Of the 4 cases of IBM examined, 3 had the characteristic 16-20 nm tubulo-filamentous inclusions, predominantly present within the cytoplasm (Figure 50). A further case (number 8) also had fine (6 nm) "actin-like" filamentous material present within multiple nuclei of one fibre (Figure 51), which have a different morphology to those of IBM. The significance of these filaments is unknown, but they have been described previously (Cullen *et al*, 1992). In one of

the 3 cases of DM tubulo-reticular inclusions were seen within the vascular endothelium (Figure 52).

Paracrystalline arrays were seen in the skeletal muscle from the coxsackie A21 infected mouse (Figure 53), however, they were difficult to find and presumably most of the virions remained as free particles. Although no filamentous inclusions or tubulo-reticular structures were seen in this case, areas containing myelin figures, similar to those of IBM, were present.

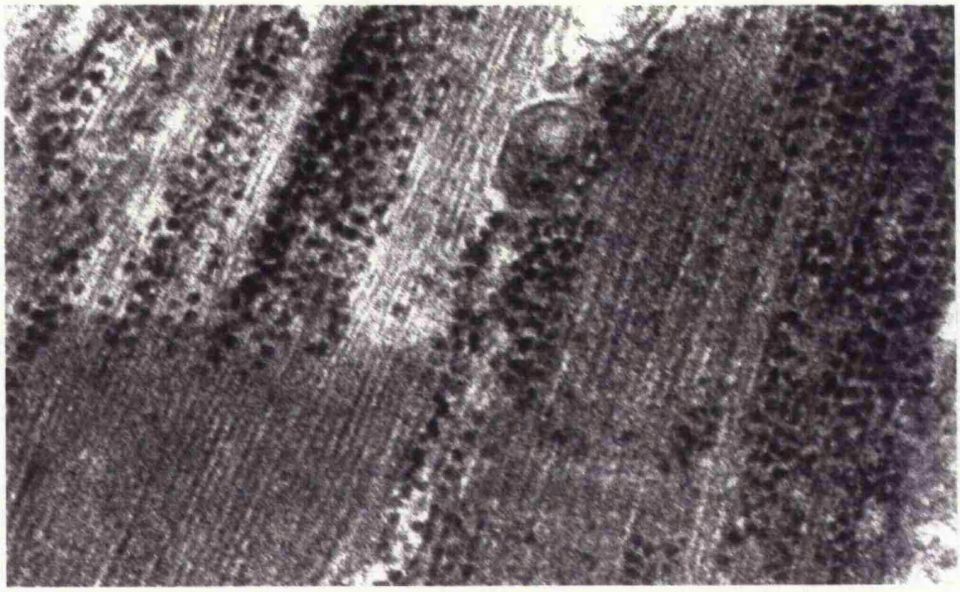


Figure 49 - Electron micrograph showing prominent glycogen from case 9 (PM). [Magnification X 105,000]

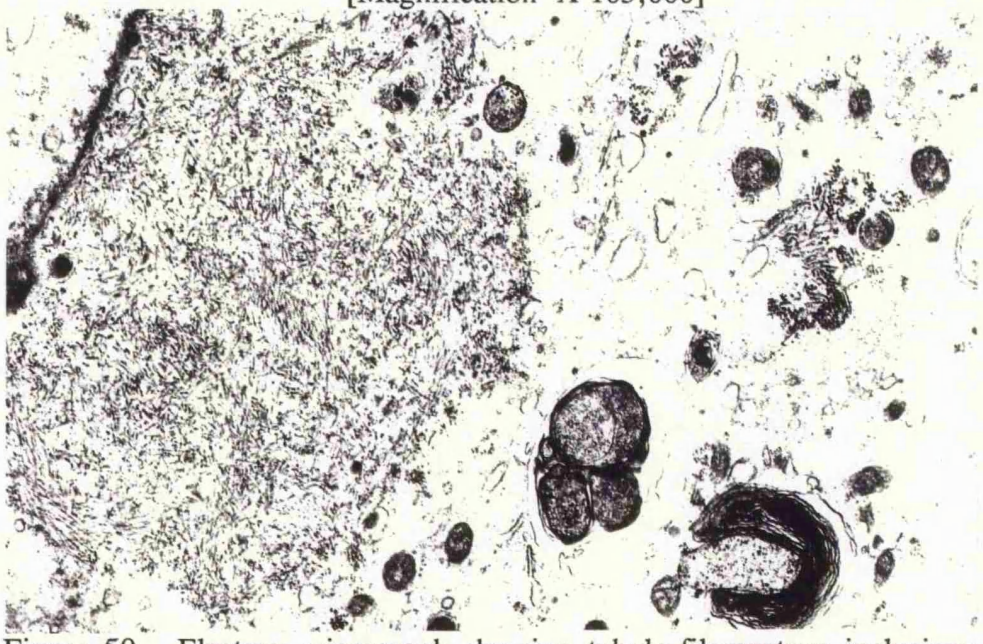


Figure 50 - Electron micrograph showing tubulo-filamentous inclusions from the cytoplasm in case 16 (IBM). [Magnification X 24,000]

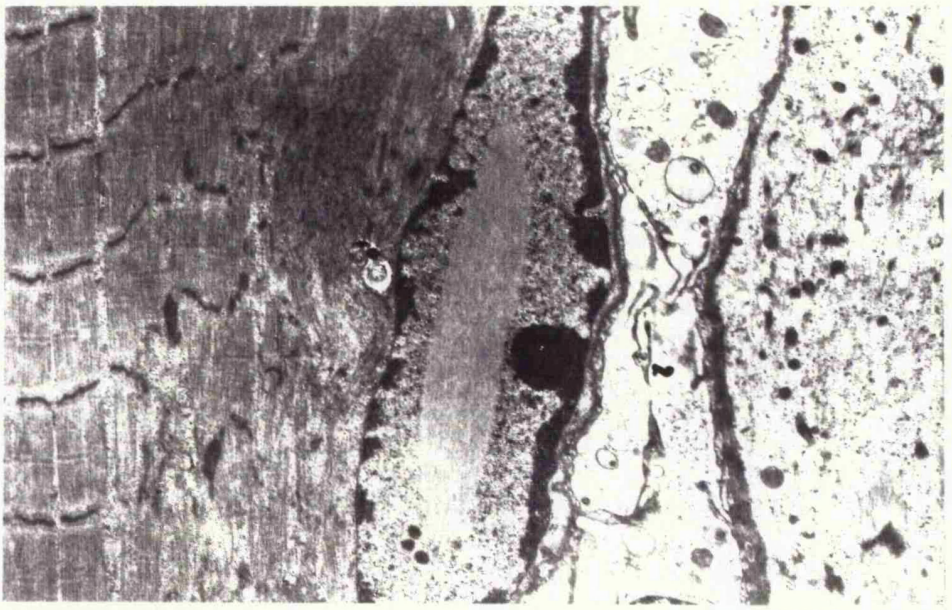


Figure 51 - Electron micrograph showing the fine intra-nuclear filaments from case 8 (PM/MCTD). [Magnification X 9,600]

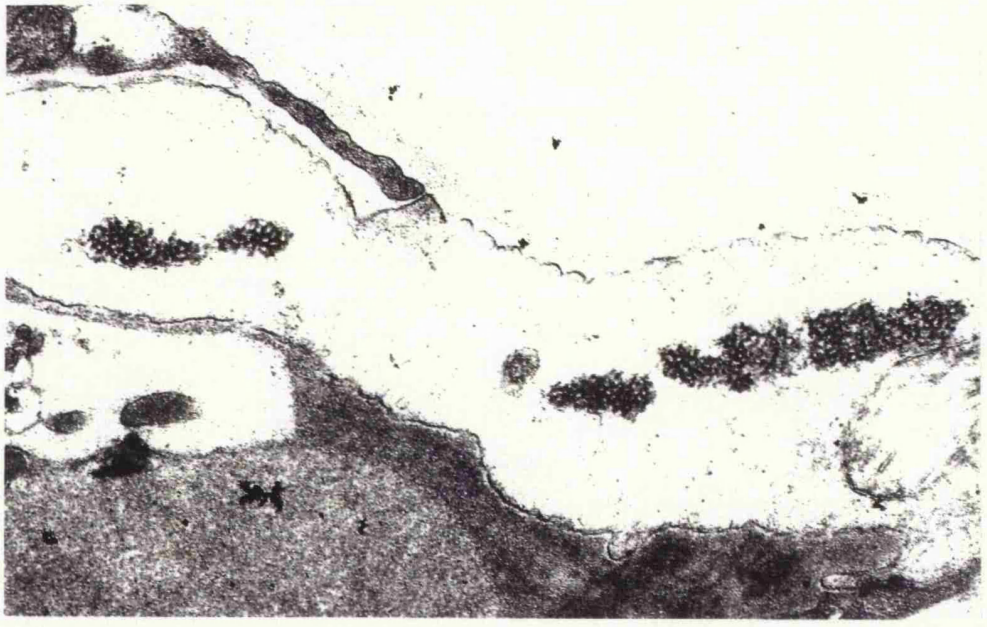


Figure 52 - Electron micrograph showing tubulo-reticular inclusions from the endothelium in case 14 (DM). [Magnification X 35,000]

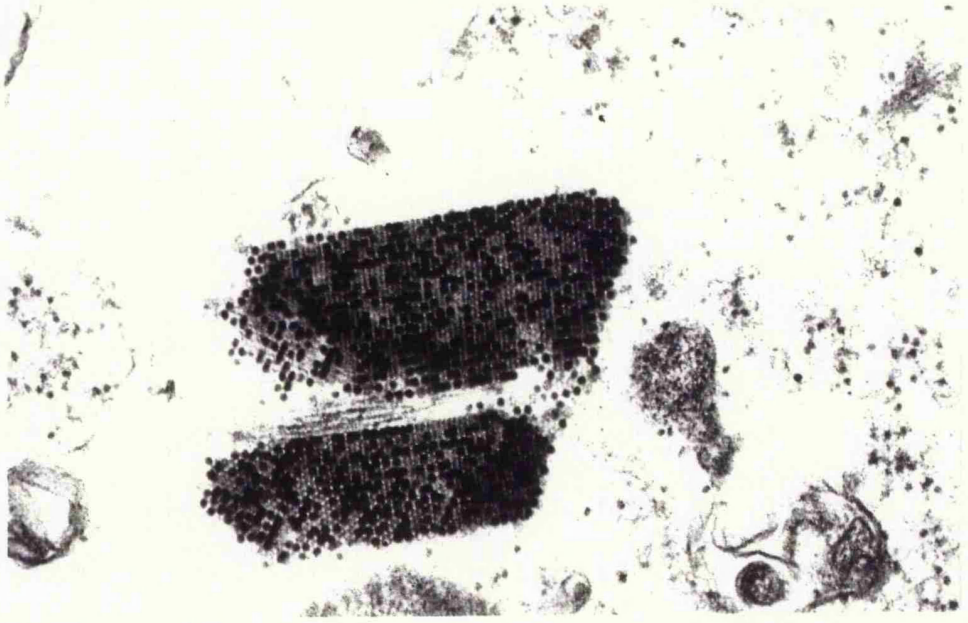


Figure 53 - Electron micrograph showing paracrystalline array within skeletal muscle from mouse infected with coxsackie A21 virus. [Magnification X 51,000]

The absence of paracrystalline arrays in these cases using electron microscopy presumable reflects the rarity of such structures in IIMD. The absence of detectable virus RNA by ISH and reverse transcription-PCR confirms that the tubulo-reticular structures, tubulo-filamentous structures and glycogen particles (which are of similar size and morphology to picornavirus particles) are not of picornavirus origin.

PART 5

CONCLUSIONS

1. ISH on mice tissues

The results of this part of the study demonstrate the very specific manner in which *in situ* hybridisation with 30 mer oligonucleotide probes can detect RNA species at a cellular level. Both normal host RNA (mitochondrial rRNA, GAPDH mRNA) and foreign virus RNA (coxsackie A and B strains) can be detected within formalin-fixed paraffin embedded tissues. The use of short oligonucleotide probes allows good tissue penetration without excessive proteinase digestion, thereby maintaining excellent tissue morphology. The choice of digoxigenin as a hapten provided adequate sensitivity as well as low levels of background.

Non-specific signal within the keratin layer of the epidermis was seen with all the oligonucleotide probes used. However, this was easily distinguished from genuine hybridisation and could be blocked by acetylation of sections prior to hybridisation. Blocking by acetylation suggests that keratin binding is likely to be a charge related phenomenon.

Table 8, showing the results of using the coxsackie oligonucleotide probes separately with each coxsackie virus strain (section 3.4.5) shows the high degree of conservation of the coxsackie virus genome, in the sequences selected to be used as probes. All strains of virus examined were detected by the "cocktail" of coxsackie oligonucleotides, including many strains for which the sequence is still unknown. These findings suggest that the sequences selected for use as probes, most of which are within the 5'UTR, have an important function. The 5'UTR has a complex secondary structure and appears to be involved in interaction with the host ribosomal machinery (see section 2.3.3). Presumably these conserved sequences are essential for normal interaction with host rRNA and therefore translation of virus RNA. The main exception to the sequence conservation is coxsackie A21, which may be more closely related to polio viruses than coxsackie B viruses (Hughes *et al*, 1989), and is only recognised by 2 of the coxsackie oligonucleotides (CXB1 and 4). These oligonucleotides have 3-5 base pair mismatches with the published sequence for coxsackie A21 virus and if higher conditions of stringency for hybridisation and post-hybridisation washes (i.e. 50% compared with 30% formamide), neither oligonucleotide detected coxsackie A21. These results demonstrate the high degree of specificity possible with short oligonucleotide probes under the conditions of hybridisation used for this study. The absence of any signal with probe B/ECHO suggest that with low GC content (<30% GC rich) oligonucleotides fewer than 4

base pair mismatches are needed for hybridisation to occur, even at conditions of low stringency (i.e. 0% formamide).

One oligonucleotide (CXB6), not from the 5'UTR, appeared to be group B specific (of those virus strains examined), but it is likely from published sequence data that coxsackie A9 would also be detected. Also the Echo viruses, which are closely related to coxsackie B viruses (Auvinen *et al*, 1989), may also be detected by this oligonucleotide.

The distribution of virus RNA in mice sections correlates well with the tissues showing morphological evidence of damage. Two patterns of distribution were seen with the coxsackie A virus strains showing diffuse involvement of skeletal muscle including the tongue and diaphragm and focal involvement of brown fat. Coxsackie B virus strains showed a more focal distribution within skeletal muscle, selectively within the front limb-girdles and head/scalp musculature, but no involvement of the tongue, diaphragm or hind limb-girdle. The distribution within brown fat was in the periphery of lobules, with additional involvement of salivary gland, pancreas and thyroid. The group B strains all showed CNS involvement with virus present in the anterior horn cell regions of thoracic and cervical spinal cord, neurons within the brainstem and occasional cortical neurons. Of the group A strains only coxsackie A16 showed any CNS involvement, which was in the same distribution as that seen with group B viruses. These differing pattern of distribution between the two groups is likely to be due to binding to different receptors, although the possibility of different cellular factors being involved cannot be discounted.

The finding of coxsackie B group viruses within the CNS is of particular interest because of the suspected association between motor neuron disease (MND) and coxsackie viruses. There have been two serological studies which provide evidence for an association between coxsackie B group viruses and patients with MND. In the first study (Kennedy *et al*, 1988), 71% of patients with MND had raised neutralising antibodies to coxsackie B group viruses, compared to 20% of controls. The second study (Bartfeld *et al,* 1989) demonstrated the presence of circulating immune complexes in 60% of patients that, after injection into rabbits, induced antisera which reacted specifically with enterovirus infected cells. Furthermore, peripheral lymphocytes from 55% patients, but only 5% of controls, produced significant amounts of lymphokines in response to extracts from coxsackie B4-infected cells. The results therefore support this hypothesis by confirming the tropism of coxsackie viruses for the sites affected by MND.

2. ISH on IIMD cases

Using control probes for poly(A) tails, GAPDH mRNA, kappa and lambda light chain mRNA and mitochondrial rRNA it was possible to demonstrate RNA preservation within tissues. Probes to poly(A) produced a signal of adequate strength, but because the signal was diffuse within the cytoplasm of all cells it was not as easy to distinguish from background signal as the probes to mitochondrial rRNA which produced discrete granular staining. The mitochondrial rRNA probes also had the advantage of being approximately 50% GC rich (compared to 0% for the probes to poly-A tails), so could be used under the same conditions of stringency as the coxsackie virus probes. The light chain probes produced strong signal within plasma cells, but not all cases of IIMD had plasma cells present. The GAPDH probes did not produce signal of enough strength to be of use in the human cases.

With mitochondrial rRNA probes all cases had evidence of RNA preservation, although in some of the larger blocks of formalin-fixed muscle signal intensity was greater around the periphery of the sections. This is presumably due to more rapid penetration of fixative preventing RNA degradation. The frozen blocks showed a more uniform pattern of RNA preservation, although great care had to be taken to prevent sections warming up prior to fixation in cold paraformaldehyde because of the rapid degradation of RNA. All attempts to store frozen sections resulted in some loss of signal, so for this study I used sections immediately after being cut.

Using the coxsackie virus oligonucleotides produced discrete strong signal within abnormal fibres in a proportion of the cases of IIMD. However, this signal did not disappear following RNase predigestion of sections and was also seen with a number of control oligonucleotides (including probes to EBV nuclear RNA, insulin mRNA and PTH mRNA). The signal was therefore not due to hybridisation but an artefact common to all oligonucleotide probes. On review, all of these positive cases were of inclusion body myositis, and signal was seen in the same distribution as the known location of the filamentous inclusions i.e. around vacuoles and within nuclei. Such a localised form of artefact had not been seen previously within the department and despite numerous attempts could not be blocked. This type of binding has not been reported in previous *in situ* hybridisation studies which have used much larger DNA or RNA probes (Rosenberg *et al*, 1989; Yousef *et al*, 1990: Behan *et al*, 1992) and may therefore, be related to the size of the oligonucleotide probes.

The second form of artefactual probe binding seen in IIMD cases was to mast cells and could be effectively blocked by tissue acetylation, strongly suggesting that it was due to a charge related phenomenon. It seems likely that the negatively charged DNA probes bind to basic substances (such as histamine) within mast cell granules. The signal was very similar to the granular binding to mononuclear cells seen in two of the previous studies with probes to coxsackie virus and TMEV (Rosenberg *et al*, 1989; Behan *et al*, 1992), thought by these authors to be genuine signal.

The finding of such artefacts reinforces the importance of appropriate controls in all hybridisation experiments.

No genuine hybridisation of the coxsackie probes was seen in any of the cases of IIMD.

3. ISH on human myocarditis cases

The post-mortem cases of myocarditis all showed some signal with the mitochondrial rRNA oligonucleotides indicating some RNA preservation (although this was more variable than the RNA preservation of the IIMD cases). None of the cases showed any *in situ* hybridisation signal with the GAPDH oligonucleotides which may be in part due to sub-optimal RNA preservation but also GAPDH mRNA may be less highly expressed in cardiac than skeletal muscle because signal within mice myocardium was much less than in skeletal muscles.

Using the coxsackie virus oligonucleotides 2/10 cases demonstrated strong and specific signal within myofibres, predominantly in areas of inflammation and necrosis. Such a distribution suggests that lytic infection of myocardial fibres by coxsackie virus is the pathogenic mechanism involved in myocarditis in these positive cases. The distribution in one case (case 4) was sub-endocardial which is of interest because of the suspected association between coxsackie viruses and endocardial fibroelastosis in children (Fruhling *et al*, 1962).

The finding of positive cases in post-mortem tissues (where RNA preservation is generally worse that biopsy material) demonstrates the ability of *in situ* hybridisation to detect "wild type" viruses in human tissue sections. It also appeared that virus RNA was better preserved than mitochondrial rRNA because signal was strong even in areas where little or no mitochondrial rRNA signal was seen. One possible explanation for this is that much of the virus RNA is "packaged" within it's protein capsid, which is resistant to RNase activity.

4. Reverse transcription-PCR

Methods for reverse transcription-PCR were developed from previous protocols. Considerable improvements in PCR results were obtained by modifying the RNA extraction technique from that previously published by Jackson *et al* (1992). This then allowed consistent detection of viral RNA from single paraffin-embedded sections, with detection of all 11 strains of coxsackie virus used and 3 polio virus strains (which could not be detected by *in situ* hybridisation). Detection of virus was possible using only small amounts of a tissue section (0.01% tissue section extract), without using either "nested" PCR or Southern blotting, indicating the sensitivity of the technique.

Investigation of all cases of IIMD produced negative results with the coxsackie primers (in contrast to the murine and human myocarditis cases). These finding are in agreement with 2 recent studies (Leff *et al*, 1992; Leon-Monzon and Dalakas, 1992). One study has found virus present by PCR in 8/20 cases (Behan *et al*, 1992), however these authors have using the same technique have also claimed to have found coxsackie virus in cases of myalgic encephalomyelitis (as well as some controls) (Gow *et al*, 1991). After sequencing of the product from this latter study it was found that there was a high degree of homology with normal human calsequestrin mRNA and the vaccine strain of polio virus type 2 (Gow and Behan, 1991), which also questions the significance of their positive findings in IIMD.

The control primers chosen (to GAPDH mRNA) produced strong and consistent bands on RNA extracted from frozen human skeletal muscle tissue, but more variable bands were produced on extracts from formalin fixed paraffin embedded human skeletal muscle. This suggests differences in quantity and/or quality of RNA produced from the differently stored tissues. One problem with some of the smaller skeletal muscle blocks is that it was easy to lose part or all of the tissue section during the extraction procedure. Control primers produced bands in 70% of the post-mortem human myocardium cases, but only following Southern blotting and ^{32}P probing of the PCR product.

5. Comparison of ISH to reverse transcription-PCR

Reverse transcription-PCR is a more sensitive technique that ISH, especially so when either followed by Southern blotting of further rounds of "nested" PCR. However, the information obtained is much more limited and the extreme sensitivity

makes contamination by PCR product an important practical problem in it's application. ISH does not have this problem, although non-specific probe binding to tissues can also cause false positive results if the appropriate controls are not performed. ISH also provides much more information by providing the precise cellular location of the target nucleic acid and allowing this to be correlated with the morphological changes (as in the myocarditis cases). Both techniques are, however, complimentary when used together.

6. Ultrastructural findings

No paracrystalline arrays were identified in the 14 cases of IIMD examined, although a number of other structures of possible viral origin were seen in several cases. The absence of virus using ISH and reverse transcription-PCR in the cases would suggest that these latter structures are not of coxsackie virus origin.

7. Role of coxsackie virus in IIMD

The absence of coxsackie virus RNA in any of the cases of IIMD makes it unlikely that persistent muscle infection by coxsackie virus is involved in the pathogenesis of IIMD. Lytic infection, with large amounts of virus causing cell necrosis, is clearly not the mechanism causing muscle fibre necrosis in IIMD. This is in contrast the findings in 2 of the cases of childhood myocarditis, where large amounts of viral RNA was present within cardiac myocytes, in a distribution to suggest that it is the cause of cell necrosis and the associated inflammatory cell infiltrate.

A role for coxsackie virus in the aetiology of IIMD has not, however, been excluded, and in particular the possibility of IIMD being an autoimmune disease "triggered" by coxsackie virus infection remains. A transient infection by coxsackie viruses, with involvement of skeletal muscle, could expose muscle antigens usually "hidden" from view by the immune system and lead to the production of an autoimmune process. Interaction between virus and host tRNA synthetases could also cause autoantibody production (Walker and Jeffrey, 1986). Although highly speculative this hypothesis would explain the serological association between coxsackie virus and IIMD, the production of a range of myositis specific antibodies (see Table 3 and Plotz *et al*, 1989) and the response of symptoms to steroid therapy.

8. Future studies

Animal models of coxsackie virus induced myositis have not been extensively investigated. Murine myositis is known to be dependant on the presence of an intact host immune system (Ytterberg *et al*, 1987 and 1988) and recent work has shown that myositis persists long after infectious virus or virus RNA can be found in tissues (Tam *et al*, 1991; Zoll *et al*, 1993). Further investigation of this model, with particular emphasis on the immunological factors involved, may lead to a better understanding of human myositis. Funds are currently being sought to pursue this, as well as to apply the techniques developed in this study (ISH and reverse transcription-PCR) to investigate the presence of coxsackie virus RNA in human tissue from cases of motor neuron disease.

In collaboration with Dr A Foulis (Glasgow) the presence of coxsackie virus RNA in the pancreases fron cases of type I diabetes mellitus is being investigated.

APPENDIX 1 - REAGENTS

Aminoalkylsilane coating of slides

1. Immerse slides in 1% Lipsol. (30 minutes)
2. Wash in running tap water. (30 minutes)
3. Wash in ultrapure water. (2 x 5 minutes)
4. Wash in 95% IMS. (2 x 5 minutes)
5. Air dry in front of fan. (10 minutes)
6. Coat slides in a freshly prepared 2% solution of 3aminopropyltriethoxysilane (Sigma A3648). (5 seconds)
7. Rinse twice in dry acetone.
8. Wash twice in ultrapure water.
9. Air dry at 42°C overnight.
10. Store at room temperature in dust free environment.

Blocking solution to block non-specific binding prior to adding antibody during ISH.

Dissolve 3g of bovine serum albumin in 100ml of TBS and add 100 µl of Triton X. Filter through Whatman filter paper and store at 4°C until use.

BCIP solution for detection of alkaline phosphatase

1. Weigh 50 mg of 5-bromo-4-chloro-3-indolyl phosphate into a bijoux.
2. Add 1 ml of dimethyl formamide in a fume cupboard.
3. Dissolve and store in the dark at 4°C.

Buffer 3 for the detection of alkaline phosphatase.

Add: 10 ml 1M Tris Cl, pH 9.5, 2 ml 5M NaCl and 5 ml 1M $MgCl_2$ to 83 ml of sterile ultrapure water.

Denaturing solution for Southern blotting (1.5 M NaCl/0.5 M NaOH)

1. Add 175.32 g NaCl and 40 g NaOH to 1400 ml ultrapure water.
2. Dissolve and add further water to a final volume of 2 1.
3. Store at room temperature in a winchester bottle.

Dextran sulphate (50%)

1. Dissolve 25 g dextran sulphate (Sigma D6001) in 50 ml of DEPC treated water, at 65°C, using RNase free glassware.
2. Store at 4°C.

DEPC water

1. Fill empty clean 2 litre bottle with ultrapure water.
2. Add 2 ml of diethyl pyrocarbonate (Sigma D.5758) in a fume cupboard.
3. Mix vigorously until DEPC globules disappear.
4. Leave to stand for at least 1 hour.
5. Autoclave.
6. Store at room temperature.

DTT

For 1 M solution dissolve 77mg dithiothreitol in 0.5ml 0.01 M sodium acetate (pH 5.2).

Ethidium Bromide (10 mg /ml)

1. Mix 0.2 g ethidium bromide in 20 ml sterile ultrapure water.
2. Wrap in silver foil and store at 4°C.

EDTA (0.5 M)

1. Weigh out 186.12 g of analar grade ethylenediaminetetra-acetic acid (disodium salt, BDH 10093).
2. Dissolve into 500 ml ultrapure water in fume cupboard.
3. Add 5 M NaOH to aid dissolution and make a final pH of 8.0.
4. Add further ultrapure water to a final volume of 1 litre.
5. Autoclave and store at room temperature.

Levamisole (1 M)

1. Dissolve 240 mg levamisole (Sigma, L9756) with 1 ml sterile ultrapure water in a bijoux.
2. Aliquot and store at -20°C.

Loading buffer for PCR products (10 X)

Bromophenol blue	2mg
50 x TAE	2ml
Glycerol	14 ml
ultrapure water	4 ml

MgCl (1 M)

1. Dissolve 20.33 g of $MgCl_2$ into final volume of 100 ml of sterile ultrapure water.
2. Autoclave and store at room temperature.

NaCl (1 or 5 M)

1. Add either 5.88 g (1 M) or 29.2 g (5 M) NaCl to 100 ml (final volume) DEPC treated water.
2. Autoclave and store at room temperature.

Neutralising solution for Southern blotting (1.5 M NaCl/1 M Tris HCl pH 8.0)

1. Add 175.32 g NaCl and 242.2 g Tris to 1200 ml ultrapure water.
2. Dissolve on a spinmix in a fume cupboard.
3. Adjust pH to 8.0 with approximately 100 ml concentrated HCl.
4. Make up to 2 1 with water and store at room temperature in a winchester bottle.

NBT solution for detection of alkaline phosphatase

1. Weigh 75 mg of nitroblue tetrazolium into a bijoux bottle.
2. Add 700 µl of dimethyl formamide in a fume cupboard and 300 µl of sterile water.
3. Dissolve and store in the dark at 4°C.

Phosphate buffered saline (PBS)

1. Dissolve 8.5 g NaCl, 1.07g of anhydrous Na_2HPO_4 and 0.39 g $NaH_2PO_42H_2O$ in 700ml ultrapure water.
2. Make up to 1 litre with further water.
3. Autoclave and store at room temperature.

PE for Southern blotting (5 X)

	Weight	Final concentration
Tris	14.14 g	250 mM
Sodium pyrophosphate	2.5 g	0.51%
SDS	25 g	5%
Poly vinyl pyrrolidone	5 g	1%
Ficoll	5 g	1%
EDTA	4.653g	25 mM

1. Dissolve Tris and EDTA in 350 ml sterile water.
2. Adjust pH to 7.5 with HCl.
3. Add other reagents.
4. Heat to 65°C then cool to 37°C.
5. Dissolve 5 g bovine serum albumin in 50 ml sterile water.
6. Add to other reagents and make final volume 500 ml with sterile water.
7. Heat to 65°C for 15 minutes and store at room temperature.

PE, modified for ISH (10 X)

1. Add 30.29 g Tris (hydroxymethyl) methylamine and 9.31 g EDTA to 350 ml DEPC treated water in fume cupboard.
2. Adjust pH to 7.5 with concentrated HCl.
3. Add :
   5 g tetra sodium pyrophosphate (Sigma)
   10 g poly vinyl pyrrolidone (Sigma)
   10 g Ficoll (Sigma)
4. If necessary heat to 65°C to dissolve.
5. Add further DEPC treated water to make final volume 500 ml.
6., Store at room temperature.

Post-hybridisation washes for Southern blotting

	4X	2X
Formamide	60ml	60ml
20 X SSC	40ml	20ml
UP water	100ml	120ml
10% SDS	2ml	2ml
TOTAL	200ml	200ml

Proteinase K (1mg/ml)

1. Weigh out 5 mg proteinase k (Boehringer/1.000.144) into an RNase free bijoux bottle.
2. Add 5 ml 0.05 M Tris pH 7.65.
3. Split into 100 µl aliquots in sterile 0.5 ml eppendorfs.
4. Store -20°C.

RNase A (10 mg/ml)

1. Mix the following in a sterile bijoux bottle:
   10 mg pancreatic RNase
   10 µl 1 M Tris HCl pH 7.5
   15 µl 1 M NaCl
   9.75 µl sterile ultrapure water.
2. Aliquot and heat to 70°C for 15 minutes, to destroy DNase activity.
3. Store at -20°C.

Salmon sperm DNA (10 mg/ml)

1. Using forceps add 0.5 g deoxyribonucleic acid from salmon testes to a 100 ml beaker (RNase free).
2. Add DEPC treated water to the 50 ml line.
3. Add small flea and seal with nescofilm. Dissolve overnight on the spinmix at 4°C.
4. Embed beaker in bucket of ice and sonicate for 30 x 30 second bursts at 15 amplitude-microns.
5. Dispense into 1 ml aliquots in eppendorfs.
6. Pierce lids with sterile needle and boil for 10 minutes to denature.
7. Cool and store at -20°C.

SDS (10%)

1. Add 100 g sodium dodecyl sulphate (launyl sulphate) to a 1 litre beaker (RNase free) in the fume cupboard.
2. Add DEPC treated water to 800 ml level.
3. Cover with nescofilm over the top and dissolve using a spinmix.
4. Store at room temperature.

Solution D for RNA extraction

25 ml stock solution
0.18 ml 2-mercaptoethanol
n.b. solution stable for 1 month.

SSC (20X)

1. Dissolve 877 g NaCl (analar) and 441 g trisodium citrate dihydrate (analar) in 4 litres DEPC treated water.
2. Adjust pH to 7 with a small amount of 0.1 M HCl.
3. Add further DEPC treated water to make up to 5 litres.
4. Filter, autoclave and store at room temperature.

Stock solution for RNA extraction

4 M guanidinium thiocyanate
25 mM sodium citrate pH 7
0.5% sarcosyl
n.b. solution stable for 3 months

Tris-acetate-EDTA concentrate (50X)

1. Add 484 g Tris (hydroxymethyl) methylamine to 200 ml ultrapure water.
2. Dissolve in fume cupboard on a spinmix.
3. Add 200 ml of 0.5 M EDTA.
4. Add 114.2 ml glacial acetic acid.
5. Mix and make up final volume to 2 litres with ultrapure water.
6. Store at room temperature in a winchester bottle.

Tris buffered saline concentrate (20 X)

1. Dissolve 60.57 g Tris (hydroxymethyl) methylamine and 87.66 g NaCl in 350 ml sterile ultrapure water in the fume cupboard.
2. Adjust the pH to 7.65 with concentrated HCl.
3. Make up to a final volume of 500 ml with sterile ultrapure water.

For use as Tris buffered saline at 0.05 M Tris and 0.15 M NaCl, dilute with 9.5 litres of ultrapure water.

Tris-EDTA

1. 12.11 g of analar grade Tris is weighed and added to 50 ml of ultrapure water.
2. 3.72 g of ethylenediaminetetra-acetic acid (disodium salt, BDH 10093) is added to the solution.
3. Adjust the pH to 8.0 with HCl.
4. Add further ultrapure water to make final volume 100 ml.
5. To make 1 x TE take 1 ml of the solution and add 99 ml ultrapure water.
6. Autoclave.
7. Store at room temperature.

Tris-HCl (1 M)

1. Dissolve 121.14 g of Tris (hydroxymethyl) methylamine in 500 ml of DEPC treated water.
2. Transfer to fume cupboard and adjust pH as required with concentrated HCl.
3. Make up volume to 1 litre with DEPC treated water.
4. Autoclave and store at room temperature.

APPENDIX 2 - PROTOCOLS

Actetylation step to block non-specific binding in ISH

After sections have been post-fixed in paraformaldehyde:

1. Rinse in DEPC treated water for 5 minutes.
2. Dehydrate in 95% and 99% IMS - 2 X 2 minutes.
3. Place in freshly prepared 0.25% acetic anhydride in xylene for 5 minutes.
4. Place in 99% then 95% IMS - 2 X 2 minutes.
5. Rinse in DEPC treated water for 5 minutes.

Continue ISH by adding pre-hybridisation solution.

Buffer for standard DNA PCR

Reagent	Volume	Final concentration
1M Tris Cl (pH 8.8)	450µl	45mM
1M $(NH_4)_2SO_4$	110µl	11mM
1M $MgCl_2$	45µl	4.5mM
10mM dNTPs	50µl	50µM each
20mg/ml BSA	55µl	110µg/ml
Sterile H_2O	5.29ml	

Volumes given for 10 aliquots of 20 reaction volumes. Use 30 µl in a final reaction volume of 50 µl.

Buffers for reverse transcription-PCR

Reverse transcription step

Reagent	Volume	Final Concentration
1M Tris Cl (pH 8.4)	112µl	45mM
1M $MgCl_2$	5µl	2mM
1M $(NH_4)_2SO_4$	26µl	11mM
10 mM dNTP	60µl	250uM each
20mg/ml BSA	13µl	110ug/ml
DEPC water	1459µl	

PCR step

Reagent	Volume	Final concentration
1M Tris Cl (pH 8.8)	225µl	45mM
1M $(NH_4)_2SO_4$	55µl	11mM
1M $MgCl_2$	27µl	4.5mM
20mg/ml BSA	28µl	110µg/ml
Sterile H_2O	3.73ml	

Use 13µl reverse transcription buffer in a volume of 20µl for reverse transcription, then add 23µl of PCR buffer to make a volume of 50µl for the PCR.

In situ hybridisation using digoxigenin labelled synthetic oligonucleotides

1. Dewax sections (4μm on silane coated slides) with xylene/graded alcohols.
2. Rinse (DEPC treated water).
3. 2x SSC, 70°C for 10 minutes.
4. Rinse (DEPC treated water).
5. 0.05M Tris, pH 7.65 for 2 minutes.
6. Proteinase K (5-10 μg/ml in 0.05M Tris) for lhr at 37°C.
7. Rinse (DEPC treated water).
8. 1 X PBS for 2 minutes.
9. 0.4% paraformaldehyde in 1 X PBS for 20 minutes at 4°C.
10. Rinse (DEPC treated water).
11. Prehybridisation solution, 200 μl/section, lhr at 37°C (30% formamide, 600mM NaCl, 1 X PE, 10% dextran sulphate, 150μl/ml SSDNA).

e.g. for 6ml: 1590μl water
720μl 5M NaCl
600μl 10 X PE
1200μl 50% dextran sulphate
90μl 10mg/ml SSDNA (boil 5 minutes, chill on ice)
1800μl 100% formamide

12. Hybridisation solution, 50μl /section, overnight with coverslip at 37°C (add probe to the prehybridisation solution to make it at 0.1 ng/μl).

N.B. all glassware and solutions up to this stage are treated with diethyl pyrocarbonate (sigma)-add 1ml to 1 litre of solution, leave overnight, then autoclave).

13.2 X SSC/30% formamide 37°C , 3 X 5 minutes washes.
14. Rinse (ultrapure water).
15. Block at RT for 10 minutes (100ml TBS, 3g BSA, 100μl triton X).
16. Antidigoxigenin alkaline phosphatase (Boehringer Manheim) at 1:600 in blocking solution, 30 minutes on rocker.
17. Rinse TBS.
18. Rinse UP water.
19. Buffer 3, 5 minutes (10ml 1M Tris Cl pH 9.5, 5ml lM $MgCl_2$, 2ml 5M NaCl, 83ml UP water).

20. Substrate (2ml buffer 3, 2 µl 1M levamisole. 8.8 µl NBT, 6.6 µl BCIP), 100 µl/section, coverslip and leave in dark for 1-16 hrs.
21. Rinse and counterstain with haematoxylin.
22. Mount in Apathy's.

N.B. If using a probe with low GC content e.g. oligo.dT, then replace formamide with water at all stages.

Ethanol precipitation of oligonucleotides

1. Add 1/10 volume of lM NaCl to the oligonucleotide solution.
2. Add 3 X volume cold absolute ethanol.
3. Mix.
4. Leave at -70°C for at least 30 minutes.
5. Microfuge at 4°C , 13,000 RPM for 10 minutes.
6. Tip off ethanol and blot dry.
7. Dry in vacuum dessicator for 15 minutes.
8. Re-suspend in 1 X TE.

Probe labelling 1 - standard homopolymer tailing method

Add the following in an eppendorf tube:

1. Sterile water - 64 µl
2. Oligonucleotide (200ng) - 2 µl
3. $CoCl_2$ (10mM) - 10 µl
4. Sodium Cacodylate (2.8 M) - 5 µl
5. 1 M Tris pH 6.8 - 3 µl
6. 1 mM digoxigenin-11-dUTP - 2.3 µl
7. 1 mM dCTP - 4.7 µl *
8. 2 mM DTT - 5 µl
9. TdT - 4 µl

Mix and incubate for 2 1/2 hours at 37°C then separate in spun column by adding 1 µl 10% SDS to 100 µl probe.
* this was later omitted

Probe labelling 2 - using chemical linkage to amino groups

The oligonucleotides are labelled and purified as above only using 30 µl 0.4 mM alyll amine dUTP instead of the above nucleotides, (reducing the amount of water to keep the total volume to 100 µl).

1. Once passed through a spun column 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1) is added.
2. Invert and mix.
3. Microfuge for 1 minute at 1300 RPM.
4. Take off the aqueous (upper) layer and pass through a spun column.
5. Add 16 µl of digoxigenin NHS in DMF (0.5 mg in 75 µl DMF).
6. Add 20 µl 1M sodium borate.
7. Add 65 µl sterile water.
8. Leave over night at room temperature.
9. Separate in 2 spun columns.
10 Aliquot and freeze.

Probe labelling 3 - using kit provided by Boehringer Mannheim

Add the following in an eppendorf tube:
1. Sterile ultrapure water - 68 µl
2. Oligonucleotide (200 ng - 1 µg) - 10 µl
3. 10 mM $MnCl_2$ - 10 µl
4. 5 x reaction buffer - 20 µl
5. 1mM digoxigenin-11-dUTP - 2.5 µl
6. TdT - 1 µl

Mix and incubate at 37°C for 2 1/2 hours then separate in spun columns as above.

RNase pre-treatment for ISH

After proteinase K digestion:

1. Rinse slides in DEPC water thoroughly - 2 X 5 minutes.
2. Place slides in a humidification chamber and cover with 100 µg/ml RNase A (diluted in a buffer containing 2 X SSC/10 mM $MgCl_2$).
3. Incubate at 37°C for 1 hour.
4. Rinse slides in DEPC water.

(n.b. all sections treated with RNase should be kept separate from the rest, and not placed in the RNase free glassware).

Continue ISH by proceeding to paraformaldehyde step.

RNA extraction for reverse transcription-PCR (modified for fixed tissue)*

1. 10 μm paraffin section into eppendorf
2. Dewax in xylene (2 X 5 mins)
3. 99% IMS (2 X 2 mins)
4. Vacuum dry 15 mins
5. Add 100 μl proteinase k (500μg/ml) and 10μl 10% SDS and incubate O/N 37°C
6. Add: 100μl solution D
   10μl 2M sodium acetate pH 4
   100μl water saturated phenol
   20μl chloroform:isoamyl alcohol (49:1)
7. Mix
8. Put on ice 15 mins
9. Centrifuge at 10,000 G, 20 mins, 4°C
10. Separate aqueous phase into a clean eppendorf and add equal volume isopropanol
11. Mix
12. Leave at -20°C, 60 mins
13. 10,000G, 20 mins, 4°C
14. Blot dry
15. Re-dissolve pellet in 100μl solution D and add 100μl isopropanol
16. Mix
17. -20°C, 60 mins
18. 10,000G, 20 mins, 4°C.
19. Blot dry
20. Re-suspend in 75% ethanol (100μl)
21. -20°C 60 mins
22. 10,000G, 20 mins, 4°C
23. Blot and vacuum dry
24. Re-suspend RNA pellet in DEPC treated water

*For fresh tissue add sections/minced tissue directly to solution D (step 6)

Reverse transcription - polymerase chain reaction

RNA Denaturation

1. RNA extract (from 4 X 10 μm sections) in DEPC water
2. Heat to 95°C in a water bath 10 mins then place on ice

Reverse Transcription

Mix;
1. 13μl RT buffer (45mM Tris-pH 8.4, 11 mM ammonium sulphate, 2mM magnesium chloride, 250 μM each dNTP, 110 μg/μl BSA in DEPC treated water)
2. 5μl primer 2 i.e. downstream primer (50 pmoles)
3. 1μl placental RNase inhibitor, 1:10 dilution (4 units)
4. 1μl denatured RNA (10% extract)
5. 1μl AMV Reverse transcriptase, 1:10 dilution (2.5 units)

Place in oven at 37°C for 1 hour

PCR

Add:
1. 23μl PCR buffer (45mM Tris pH 8.8, 11mM ammonium sulphate, 4.5 mM magnesium chloride, 110 μg/μl BSA in sterile water)
2. 5μl primer 1 i.e. upstream primer (50 pmoles)
3. 50μl paraffin oil to cover

Then:
Denature Cycle (96°C , 10 mins; 62 °C , 10 mins; 72°C 2 mins)
Add:
4. 1μl Taq DNA polymerase, 1:10 dilution (0.5 unit)
Then:
Thermocycle file (40 cycles: 96°C, 30 seconds; 62°C, 30 seconds, 72°C, 1 min 30 seconds)
Final extension (70°C , 10 mins, 62°C , 10 mins)
Soak file (4°C - indefinite period)

Agarose gel

1. Make up gel by dissolving 3 % Nusieve/ 1% Seakem agarose in 1 X TAE buffer
2. Run 40.5 µl of product in gel with 4.5 µl 10 X loading buffer after heating for 10 mins at 65 ^{o}C
3. Visualise with ethidium bromide 1 hr (50 µl Ethidium Bromide solution - 10mg/ml into 250ml TAE)

Southern blotting of PCR products

1. Rinse gel in UP water.
2. Denaturation buffer (0.5M NaOH/1.5M NaCl) - 30 mins.
3. Rinse in UP water.
4. Neutralising buffer (1M Tris pH 8/1.5M NaCl) - 2 X 15 mins.
5. Rinse in UP water.

Assemble Blotting Apparatus
Fill staining tray with 20 X SSC.
Make wick with 4 strips of 3MM, soaked in 20 X SSC.
Arrange on platform, flatten and cover with saran.
Draw size of gel on saran, hybond N and 3 strips of 3MM.

6. Place gel face down on hole in saran and cover with hybond and 3MM.
7. Roll flat and place paper towels on top with 1kg weight.
8. Change towels every 5 mins for 30 mins, then every 30 mins for 2 hours.
9. Dry hybond (80^{o}C , 5 mins).
10. Wrap in saran and X-link on UV transilluminator for 30 seconds.
11. Restain gel to check transfer.

Probing filter with ^{32}P labelled oligonucleotides

Prehybridisation solution

30% Formamide	Formamide	6	12	18 ml
600mM NaCl	5M NaCl	2.4	4.8	7.2 ml
10% Dextran Sulphate	50% Dextran Sulphate	4	8	12 ml
1 X PE (unmodified)	5 X PE	4	8	12 ml
150µg/ml SSDNA	10mg/ml DNA	0.3	0.6	0.8 ml
	sterile water	3.6	7.2	10.8 ml
	Total volume	20	40	60 ml

1. Warm prehybridisation solution to 37°C in oven using humidification chamber.
2. Float hybond in solution for 1 hour.
3. Add labelled probe (25ng/20ml prehybridisation solution).
4. Hybridise over night at 37°C .
5. Wash off unlabelled probe with:

    4 X SSC/30% Formamide/0.1% SDS - 2 X 10 mins at 37°C
    2 X SSC/30% Formamide/0.1% SDS - 2 X 10 mins at 37°C

6. Blot dry hybond and wrap in saran.
7. Expose to X Ray film 2 hours - 5 days.

Spun column preparation for synthetic oligonucleotide purification

1. A column is prepared in a sterile 1ml syringe plugged with a small amount polyallomer wool by filling it with a slurry of sephadex G5O(Pharmacia 17-0045-01) pre-swollen in 1 X TE, pH 8.0.
2. The column is placed in a polypropylene centrifuge tube and centrifuge at 1600g for 5 minutes, then more sephadex is added with a sterile Pasteur pipette, being careful not to introduce air bubbles which may become trapped, until it reaches the 0.9ml level.
3. The column is then equilibrated with 1 X TE/0.1%SDS, by adding 100 µl , centrifuging and measuring the effluent.
4. Once 100 µl of buffer is produced from the column (usually after 3 spins) the column is ready for use.

Labelled probe (100 µl) can now be added to the top of the column which is placed over a clean eppendorf, in an unused polypropylene tube and then centrifuged again. Only labelled probe is produced from the column, all the smaller molecules, including unincorporated nucleotides, remain trapped within the sephadex beads.

Test strips for digoxigenin labelled oligonucleotides

1. A diluent is prepared by adding 300 µl 20 X SSC, 20 µl 10mg/ml SSDNA and 680 µl sterile water.
2. This is boiled and chilled on ice.
3. The oligonucleotide is diluted to 1ng/µl, 100pg/µl , 50 pg/µl , 10 pg/µl , 1 pg/µ l and a final solution of pure diluent is used.
4. 1 µl of each dilution is spotted on to strips of nitrocellulose at 1 cm intervals (care should be taken to avoid touching the strip with your skin).
5. The oligonucleotide is baked on to the nitrocellulose at 80°C for 2 hours, between 3MM paper.
6. The nitrocellulose is re-hydrated in ultrapure water then immersed in prewarmed blocking solution (3% BSA, 0.1% Triton X in TBS) at 42°C for 20 minutes.
7. The nitrocellulose is then baked at 80°C for 20 minutes between 3MM paper.
8. This is then re-hydrated in ultrapure water and immersed in blocking solution for 10 minutes.
9. Anti-digoxigenin antibody (conjugated to alkaline phosphatase Boehringer Manheim), is then added at a concentration of 1:600 in blocking solution for 30 minutes.
10. Unbound antibody is washed off with blocking solution (2 X 5 minutes) and ultrapure water (2 X 5 minutes).
11. The nitrocellulose is immersed in Buffer 3 for 5 minutes, then the alkaline phosphatase is visualised by adding a substrate mixture containing:

10 ml Buffer 3
20 µl 1M Levamisole
88 µl NBT
66 µl BCIP

The reaction is stopped by rinsing off the substrate after 15 minutes - 12 hours, depending on the level of signal/background.

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PUBLICATIONS ARISING FROM THIS WORK

PAPERS

1. Demonstration of the distribution of coxsackie virus RNA in neonatal mice by non-isotopic *in situ* hybridisation.
D.A.Hilton, C.Day, J.H.Pringle, A.Fletcher, S.Chambers.
Journal of Virological Methods 1992; **40**: 155-162.

2. Demonstration of coxsackie virus RNA in formalin-fixed tissue sections from cases of childhood myocarditis cases by *in situ* hybridisation and the polymerase chain reaction.
D.A.Hilton, S.Variend, J.H.Pringle.
Journal of Pathology 1993; **170**: 45-51.

3. Absence of coxsackie viruses in idiopathic inflammatory muscle disease by *in situ* hybridisation.
D.A.Hilton, J.H.Pringle, A.Fletcher.
Neuropathology and Applied Neurobiology 1994; **20(3)**: 238-242.

ABSTRACTS

1. Demonstration of coxsackie virus RNA in childhood myocarditis by *in situ* hybridisation and the polymerase chain reaction.
D.A.Hilton, S.Variend, J.H.Pringle, A.Fletcher.
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2. Absence of coxsackie virus RNA in skeletal muscle from patients with idiopathic inflammatory muscle disease.
D.A.Hilton, J.H.Pringle, A.Fletcher.
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