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Molecular detection and serotypic analysis of enterovirus RNA in archival specimens from patients with acute myocarditis

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Abstract

***Objective*—To determine whether enterovirus RNA can be demonstrated in archival necropsy material in acute myocarditis.**

***Design*—Analysis of paraffin embedded myocardial tissue from cases of acute myocarditis.**

***Setting*—University virology department.**

***Methods*—Extraction of RNA from tissue followed by polymerase chain reaction (PCR) and DNA sequence analysis.**

***Patients*—Six patients with histologically proven myocarditis and eight controls.**

***Results*—Enterovirus RNA was identified in 5 of 6 patients with myocarditis and in none of the controls. The nucleotide sequences of the PCR products showed greatest similarity to group B coxsackieviruses, particularly coxsackievirus B3.**

***Conclusion*—This study indicates that archival tissue samples, even histologically stained tissue sections, can be used to study the role of enteroviruses in myocardial disease using molecular detection techniques. If a predominant role for coxsackievirus B3 in myocarditis is confirmed by further study, this may have implications for the development of a specific vaccine.**

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Keywords: enterovirus RNA, acute myocarditis, polymerase chain reaction, nucleotide sequence analysis

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Enteroviruses are the commonest viral cause of acute myocarditis.[1] Establishing a diagnosis is not straightforward, however, as a history of a recent viral illness cannot always be obtained and histological features of endomyocardial biopsy specimens do not allow differentiation between viral and other causes of myocarditis. Furthermore, histological evidence of myocarditis may frequently be missed because of the focal nature of lesions. Traditional diagnostic methods, including virus isolation in cell culture, detection of viral antigen in biopsy or necropsy tissue, and detection of rising antibody titres, are frequently unsuccessful as cardiac symptoms usually coincide with the onset of cytotoxic T cell responses directed against viral or cellular antigens that occur late in the disease process. Although enterovirus specific immunoglobulin M (IgM) antibody may be detected in up to 50% of cases,[2–4] results must be interpreted carefully as there may be a high background prevalence of IgM responses in the general population, particularly during epidemic seasons.[5]

Such techniques as slot blot and in situ hybridisation have been employed to detect viral RNA in acute myocarditis[6–8] but are not suitable for use in diagnostic laboratories as they are too labour intensive. Reverse transcription (RT) and polymerase chain reaction (PCR) amplification of viral RNA are potentially more rapid and sensitive but have not been adequately evaluated for the diagnosis of viral myocarditis.

We have previously described a specific nested RT and PCR for the detection of enteroviral RNA in clinical samples, which is at least 10 times more sensitive than cell culture detection.[12] This paper describes how these studies have been extended to detect enteroviral RNA retrospectively in formalin fixed archival myocardial tissue including haematoxylin and eosin (H and E) stained sections. In addition, we have used nested or semi-nested RT and PCR assays to amplify three different regions of the viral genome (two in the 5′ non-translated region (5′NTR) and one in the capsid (VP2) coding region) to exclude the possibility of false positivity due to PCR product contamination. We have also sequenced PCR products derived from the 5′NTR, thereby enabling us to determine the enterovirus serotype present in heart tissue. We also detected enteroviral RNA using an in situ hybridisation protocol under evaluation in this laboratory which utilises non-isotopic probes and is therefore potentially more suitable for diagnostic use than previously described protocols employing radioactive probes requiring prolonged autoradiographic exposure. Results of these molecular investigations have been correlated with clinical findings and results of enterovirus specific IgM testing.

Materials and methods

CLINICAL SAMPLES

Postmortem heart samples were obtained from five patients with fatal myocarditis conforming with the Dallas criteria,[9] and an additional patient with giant cell myocarditis (age range from 3·5 weeks to 32 years). Table 1 gives the presenting histories of patients together with samples obtained. A comparison group con-

Table 1 Patient characteristics

Patient	Sex	Age (years)	Samples	Clinical presentation
1	M	11	Bx, FFB	4 day history of headaches and vomiting, neck pain, fever
2	F	32	FFB	Died postpartum after febrile illness
3	M	3·5 wk	H and E, FFB	Developed cardiac failure aged 11 days
4	M	21	FFB	Epigastric pain, short of breath
5	M	25	H and E	Dizzy, palpitations, falling cardiac output
6	F	25	H and E	Sore throat, lymphadenopathy rash, arthralgia, myalgia

Bx, biopsy; FFB, formalin fixed block; H and E, haematoxylin and eosin stained tissue.

Table 2 Control patients

Control	Sex	Age (years)	Samples	Diagnosis
1	M	13	FFB	Cerebrovascular accident
2	F	32	FFB	Subarachnoid haemorrhage
3	M	12 wk	H and E	Bacterial meningitis
4	M	28	FFB	Brain tumour
5	M	26	H and E	Septicaemia
6	M	35	FFB	Pneumonia
7	F	66	FFB	Cerebral infarction
8	F	42	FFB	Pancreatic carcinoma

Abbreviations as in table 1.

sisted of heart tissue from eight patients (age range 12 weeks to 66 years) who died of non-cardiological causes (table 2). Antemortem serum samples were available from five of six patients with acute myocarditis and were tested for the presence of enterovirus specific IgM.[10]

RNA EXTRACTION

H and E preparations

Slides were soaked in xylene for 48 h, immersed in ethanol for five min, and then dried in air. The coverslip was removed with a scalpel blade and the section scraped off and suspended in 100 μl of digestion buffer containing 100 mmol/l sodium chloride, 10 mmol/l TRIS hydrochloride (HCl) (pH 8·0), 25 mmol/l EDTA, 0·5% sodium dodecyl sulphate, and 0·1 mg/ml nuclease free proteinase K (Sigma; Poole, Dorset). Samples were incubated with gentle agitation at 37°C for three days after which a further 50 μg proteinase K was added to each tube and left to digest for a further 3–4 days. RNA was extracted from the supernatant using RNAzol B (Biotex, Houston, Texas) as previously described,[11] stored as dry pellets at −70°C, and reconstituted in sterile water immediately before use.

Formalin fixed paraffin embedded sections

Thick sections (10 μm) were dewaxed by washing twice in octane at room temperature for 30 min with mixing and twice in ethanol. The sections were dried at 37°C. About 10 μg tissue were suspended in 100 μl of digestion buffer containing 50 mmol/l TRIS (pH 8·0), 1 mmol/l EDTA, 0·5% Tween 20, and 10 mmol/l vanadyl ribonucleoside complexes (Gibco BRL, Paisley) with nuclease free proteinase K (Sigma) added immediately before use to a final concentration of 200 μg/ml. Samples were incubated at 55°C for 180 min, and then at 99°C for 5 min to inactivate the enzyme. RNA was extracted from the supernatant using RNAzol B.

Oligonucleotides—Table 3 shows the oligonucleotide primers used for RT and PCR. Optimal primer sequences were predicted using OLIGO 4·0 (National Biosciences Plymouth, Minnestota, USA). Primers EVU1, EVD1, EVU2, and EVD2 were designed using all published enterovirus sequences currently available and recognise highly conserved sequences within the 5'NTR of the enterovirus genome. We have shown that nested PCR using these primers is able to detect a wide range of enterovirus serotypes, including all polioviruses and group B coxsackieviruses and representative group A coxsackieviruses and echoviruses, as well as enterovirus 70.[11 12] Other primers described in table 3 were designed using published group B coxsackievirus (CVB) sequences, namely CVB1,[13] CVB3,[14] CVB4,[15] and CVB5.[16] PCR assays using these primers have been shown to detect all six CVB serotypes (not shown). Their ability to detect other enterovirus serotypes is currently under investigation.

RT and PCR amplification—Viral RNA was amplified by RT and nested or semi-nested PCR using three sets of primers to amplify distinct regions of the viral genome. Nested RT and PCR using primers EVD1/EVU1 and EVD2/EVU2 were carried out as previously described.[12] Amplification of the other two regions of the genome was carried out similarly using primers for RT and PCR and optimal magnesium chloride concentrations, primer annealing temperature, and cycle number for each primer pair as shown in table 4.

PCR contamination prevention strategy—Sections of paraffin embedded tissue were prepared using a microtome with disposable blades. A new blade was used for each block. Negative extraction and reagent controls were processed alongside each batch of test specimens to ensure that PCR contamination did not occur. Other safeguards established to minimise the risk of contamination have already been described.[11 12]

Table 3 Oligonucleotide primers used for reverse transcription and polymerase chain reaction amplification of enterovirus RNA

Enterovirus genome

5' non-translated region — *Capsid coding region*

EVU3 → *EVU5* → *EVD5* ← *EVD3* ← *EVD4* ← *EVU1* → *EVU2* → *EVD2* ← *EVD1* ← — *EVU6* → *EVU7* → *EVD6* ←

Target sequence[1]	Primer	Sequence (5' to 3')[2]	Nucleotide positions[1]
417–645	EVU1	GGTGYGAAGAGYCTAYTGAG	417–436
	EVD1	CACYGGRTGGCYAATCCA	645–628
	EVU2	CCCCTGAATGCGGCTAAT	456–473
	EVD2	ATTGTCACCATAAGCAGCCA	602–583
63–377	EVD3	CAGGCCGCCAACG	375–363
	EVU3	CGGTACCTTTGTGCGCCTGT	63– 82
	EVD4	GGCAGGCCGCCAACG	377–363
	EVU5	CCCCGGACTGAGTATC	180–195
	EVD5	AGTGCTGAGCGAAACAC	299–283
1177–1485	EVU6	SCDGGDTGGTGGTGGAA	1177–1190
	EVD6	YACYCCCATRCCRGCATT	1485–1467
	EVU7	TGGRCARAAYATGCARTAYCACTAC	1225–1249

[1] Nucleotide positions based on those of Klump *et al.*[14]
[2] Redundant base codes: Y = C or T; R = A or G; S = C or G; D = A, G or T.

Table 4 Oligonucleotide primers used for reverse transcription and polymerase chain reaction amplification of enterovirus RNA

		First PCR				Second PCR			
Target sequence	*Reverse transcription primer*	*Primers (size of product)*	*$MgCl_2$ concentration (mmol/l)*	*Annealing temperature (°C)*	*Cycle number*	*Primers (size of product)*	*$MgCl_2$ Concentration (mmol/l)*	*Annealing temperature (°C)*	*Cycle number*
417–645	EVD1	EVU1 EVD1 (229 bp)	2·0	50	30	EVD2 EVD2 (147 bp)	1·8	52	25
63–377	EVD3	EVU3 EVD4 (315 bp)	2·0	58	40	EVU5 EVD5 (120 bp)	2·4	49	15
1177–1485	EVD6	EVU6 EVD6 (309 bp)	2·6	55	40	EVU7 EVD6 (261 bp)	1·4	50	15

bp, base pairs; $MgCl_2$, magnesium chloride.

Probe preparation

A CVB3 complementary DNA clone (pCB3) and control plasmid (p2723) were used to generate enterovirus specific and control cDNA probes respectively.[17] A 6·2 kb *Kpn* I fragment and 1·0 kb *Bam* HI fragment of clone pCB3 were isolated[18] and further restricted with *Mnl* I to generate fragments of optimal length for use as in situ probes (predominantly 70–200 base pairs). DNA fragments were labelled overnight with digoxygenin employing a digoxygenin high prime labelling kit (Boehringer Mannheim Lewes, East Sussex) using 30 ng template DNA per 1·4 pmol digoxygenin-11-deoxyuridine triphosphate—that is, 4 μl labelling mix). Digoxygenin labelled DNA was precipitated, redissolved in 50 μl 0·1 mol/l Tris EDTA buffer, pH 8·0, and used at a dilution of 1:400 for in situ hybridisation. Control plasmid p2723 was restricted with *Mnl* I and labelled with digoxygenin in the same way.

In situ hybridisation

Sections (5 μm) of paraffin embedded formalin fixed myocardial tissue were mounted on silane coated slides, dried at 37°C overnight, dewaxed by immersing in two changes of xylene, and rehydrated by two successive immersions in each of 99, 95, 70, and 50% industrial methylated spirit, then in diethylpyrocarbonate treated sterile water. All subsequent pre- and post-hybridisation incubations and washing stages were carried out using the Omnislide thermal cycler and wash module (Hybaid, Teddington) except where stated. All incubations and washes were at room temperature except where stated. Nuclease- and protease-free reagents and solutions were used for prehybridisation, hybridisation, and post-hybridisation washes. Sections were washed successively in 2 × saline sodium citrate (SSC) (Sigma; 1 × SSC consists of 15 mmol/l sodium citrate and 150 mmol/l sodium chloride) for 10 min at 60°C, diethyl pyrocarbonate treated sterile water for 10 s, and then 50 mmol/l TRIS HCl (pH 7·6) (Sigma) for 5 min. Tissue sections were rendered permeable by digestion with 12·5 μg/ml proteinase K in 50 mmol/l TRIS HCl (pH 7·6) for 1 h at 37°C. Slides were then divided into two groups. One group was treated with 100 μg/ml ribonuclease (RNase) (Sigma) in 2 × SSC containing 10 mmol/l magnesium chloride for 1 h at 37°C (RNase controls). The other group was treated in RNase free buffer. The two groups of slides were washed separately in diethyl pyrocarbonate phosphate buffered saline (Sigma) for 10 s and post fixed in 4% (w/v) paraformaldehyde in diethyl pyrocarbonate treated 0·01 mol/l phosphate buffered saline at 4°C for 20 min. RNase treatment, washing, and paraformaldehyde fixation of slides were performed in baked glass chambers. Slides were then combined and washed in diethyl pyrocarbonate treated sterile water for 10 s and prehybridised using a minimal volume of hybridisation buffer (3 × SSC in 50 mmol/l TRIS HCl (pH 7·6) containing 10% dextran sulphate, 30% formamide, 150 μg/ml salmon sperm DNA, 0·1% sodium pyrophosphate, 0·2% polyvinylpyrrolidone, and 5 mmol/l EDTA) for 1 h at 37°C. Hybridisation buffer was aspirated, replaced with fresh buffer containing digoxygenin labelled enterovirus specific or control heat denatured probe at a dilution of 1:400 and slides incubated at 95°C for 5 min then 37°C overnight. Post-hybridisation washes consisted of successive washes at 37°C in 4 × SSC containing 50% formamide (twice) then 2 × SSC containing 50% formamide (twice). Sections were blocked with 5% sheep serum in modified TRIS buffered saline (50 mmol/l TRIS HCl (pH 7·6), 150 mmol/l sodium chloride, 2 mmol/l magnesium chloride, 0·1% filtered bovine serum albumin) for 1 h, drained, and incubated with alkaline phosphatase labelled anti-digoxygenin Fab antibody fragments (Boehringer Mannheim) diluted 1:600 in modified TRIS buffered saline for 1 h, then washed twice with modified TRIS buffered saline for 5 min and once with diethyl pyrocarbonate treated sterile water for 1 min. To detect bound probe, sections were covered with 0·1 mol/l TRIS HCl (pH 9·5), containing 1 mol/l sodium chloride, 1 mmol/l magnesium chloride, 1 mmol/l levamisole, 0·33 mg/mlnitroblue tetrazolium, (Boehringer Mannheim), and 0·17 mg/ml 5-bromo-4-bromo-3-indoylphosphate (Boehringer Mannheim), and incubated overnight in the dark. Slides were washed for 5 min in water, mounted in ultraviolet free aqueous mountant (RA Lamb London), and viewed by light microscopy. In some cases sections were counterstained with 1% malachite green before mounting. Specific hybridisation signals were identified as discrete areas of purplish brown colour which were

Table 5 Results of enterovirus PCR, nucleotide sequence analysis of PCR products, and enterovirus specific IgM testing in patients with acute myocarditis and in controls

No	Polymerase chain reaction			Closest sequence identity[1]	Homology[2]	IgM
	# 417–645	63–377	1177–1485			
Patient						
1	+	+	−	B3	100	X
2	+	+	+	B3	100	−
3	+	+	X	B5	97	+
4	+	+	+	B3	100	+
5	+	+	−	B3	100	+
6	−	−	X	−	−	+
Controls						
1	−	−	−	−	−	X
2	−	−	−	−	−	X
3	−	−	−	−	−	X
4	−	−	−	−	−	X
5	−	−	−	−	−	X
6	−	−	−	−	−	X
7	−	−	−	−	−	X
8	−	−	−	−	−	X

[1] Based on sequence analysis of EVU1/EVD1 nested polymerase chain reaction products.
X, not tested.

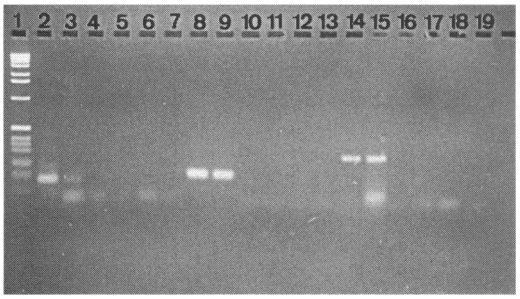

Figure 1 Enterovirus RNA in formalin fixed myocardium detected by amplification of nucleotides 63–377 (tracks 2–7), 417–645 (tracks 8–13), and 1177–1485 (tracks 14–19) of the enterovirus genome. Track 1, DNA molecular weight markers (kb ladder; GIBCO BRL). Tracks 2, 8, and 14, second polymerase chain reaction (PCR) products derived from coxsackievirus B3 (CVB3) RNA (positive contol), tracks 3, 9, and 15, second PCR products derived from patient 2, tracks 4–7, 10–13, and 16–19, PCR negative controls.

present in sections hybridised with enterovirus specific probe but were not present in replicate sections hybridised with control plasmid probe, and were either absent or greatly reduced in intensity in replicate RNase control sections.

PCR product sequence analysis

Biotinylated nested PCR products were generated for sequence analysis using primers EVD2 and EVU2 (table 3) where primer EVD2 was biotinylated. Biotinylated PCR products were captured on streptavidin coated magnetic beads (Dynal, Wirral) and denatured with 0·1 mol/l sodium hydroxide to generate single stranded DNA. This was used as template in a sequencing reaction (Autoread; Pharmacia Biotech, St Albans) using fluorescein labelled EVU2 as sequencing primer.

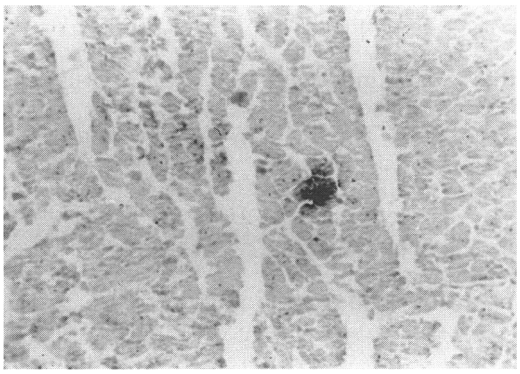

Figure 2 Enterovirus RNA detected by in situ hybridisation in myocardium from patient 2 showing a focus of virus infected myocytes.

Sequencing reactions were analysed using an ALF automated DNA sequencer (Pharmacia). Sequences were compared with published enterovirus sequences using DNASIS.

Results

Table 5 summarises the results of nested PCR amplification of RNA from heart samples from patients and controls. Enterovirus RNA was detected by PCR in five of six patients using both sets of primers in the 5'NTR, and in two of four patients using VP2 primers. Two of three H and E samples were positive by PCR as were all four paraffin embedded blocks. No enteroviral RNA was found by PCR in the comparison group. Figure 1 shows the results of PCR studies on tissue from patient No 2. Sequencing of PCR products from patients indicated that viruses detected most closely resembled CVB3 in four cases and CVB5 in one. Enterovirus RNA was also detected by in situ hybridisation in three of three patients with acute myocarditis (patient Nos 1, 2, and 4). Figure 2 shows the results of in situ hybridisation of myocardium from patient No 2. Enterovirus specific IgM was detected in four of five patients, one of whom had no detectable enteroviral RNA by PCR (No 6). Serum samples were not available from the control group for comparison.

Discussion

We have shown using nested RT and PCR, albeit on a small number of patients, that it is possible to detect enteroviral RNA in archival material collected up to 17 years before testing. This is encouraging as it is generally presumed that RNA is not very stable and cannot be detected in archival specimens. Our findings as well as those of others,[19 20] however, indicate that viral RNA is relatively stable and may be protected by the fixation process. The time interval between death and preservation of postmortem tissue may affect RNA preservation, as may the time spent in formalin before embedding.[21] As the patients were referred from different centres over a long period of time, however, it was not possible to ascertain either of these intervals.

Comparatively few virological studies have been carried out on cases of acute myocarditis employing PCR. Such studies as have been reported are based on small patient numbers.[22–25] More emphasis has been placed on investigating chronic infections, particularly dilated cardiomyopathy. This may reflect the fact that severe acute myocarditis is a relatively uncommon disease, and endomyocardial biopsy specimens and cardiac explants obtained during transplantation are only rarely available. This study used three PCR assays to amplify three distinct regions of the viral genome. In all cases where enteroviral RNA was detected, positive results were obtained in at least two of the three assays (table 5). This provides further evidence that false positivity resulting from PCR product contamination did not occur and will in due course provide

additional sequence data from the VP2 region to confirm the serotypic identity of viruses detected. The failure to detect viral RNA using primers to amplify the VP2 coding region in some patients may be due to the lower sensitivity of this assay or the greater sequence variability in this region of the genome, resulting in changes in primer recognition sequences.

The only heart sample from patients with acute myocarditis in which enteroviral RNA was not detectable by PCR (No 6) was found histologically to have giant cell myocarditis with an eosinophilic infiltrate. All the other patients had a predominantly lymphocytic infiltrate of myocardium. As yet there is no evidence implicating enteroviruses in the pathogenesis of giant cell myocarditis.

In preliminary studies we also detected enterovirus RNA by in situ hybridisation using a digoxygenin labelled CVB3 cDNA probe in three of three patients with acute myocarditis. We are currently evaluating the sensitivity and specificity of this method. In a recent study by Hilton *et al*[24] a complete correlation between in situ hybridisation and PCR was reported. As virus was detected in only two of 10 samples from patients with acute myocarditis, however, further study in larger numbers is required to provide definitive information on the relative sensitivities of PCR and in situ hybridisation. It may be possible to enhance the sensitivity of in situ detection by using in situ PCR. This would have the advantage of enhanced sensitivity while allowing localisation of enteroviral RNA in relation to the tissue architecture. This may provide information on the pathogenesis of myocardial injury

Enterovirus specific IgM responses were present in three of four patients in whom enterovirus RNA was detected in myocardium. The failure to detect an IgM response in one patient may be related to the time at which the serum was collected in relation to the disease process, or to antigenic differences between the virus that infected the patient and those used to prepare antigens for enterovirus specific IgM enzyme linked immunosorbent assay. Enterovirus specific IgM was also detected in the patient in whom enterovirus RNA was not detected in the myocardium. Although failure to detect viral RNA in myocardium could have been due to the focal distribution of virus infected cells (fig 3), this patient had giant cell myocarditis which is not known to be related to enterovirus infection. It is therefore more likely that the IgM response reflects a recent but unrelated infection.

Sequence analysis of the 5'NTR indicates that most viruses detected in myocardium showed greatest similarity to CVB3. The percentage homology between sequences of the PCR products and published sequences is high (97–100%) (table 5). This reflects the high conservation of sequence among different enterovirus serotypes in this region of the genome. The predominance of CVB3 is in agreement with another recent study on patients with myocarditis and dilated cardiomyopathy.[25] Having demonstrated that it is possible to detect enteroviral RNA from archival material, it may now be possible to conduct more extensive retrospective studies to address this issue. If these findings hold true for larger case numbers then this may have profound implications on the potential development of a vaccine specific for CVB3.

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IMAGES IN CARDIOLOGY

Hypertrophic cardiomyopathy

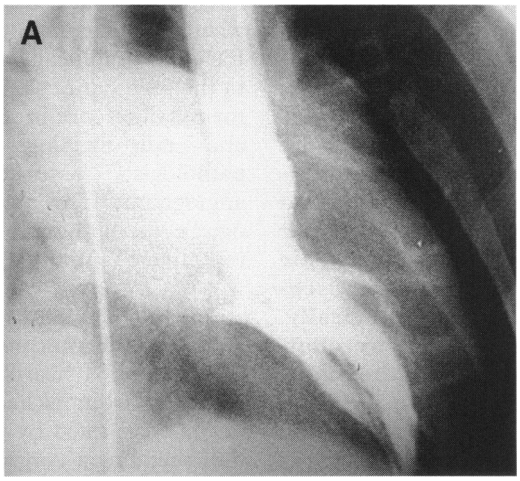

Figure A

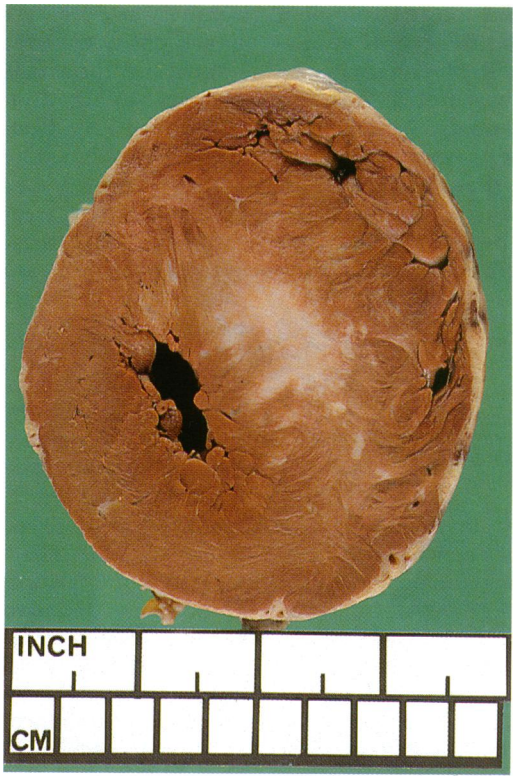

Figure B

A 60 year old woman presented with severe dyspnoea. A labile left ventricular outflow tract gradient varying between 20 and 80 mm Hg was demonstrated at cardiac catheterisation. The left ventricular angiogram (A) shows the typical appearances of hypertrophic cardio-myopathy with a very small end systolic cavity, hypertrophied papillary muscles, and associated severe mitral regurgitation.

(B) Postmortem transverse section through the heart at the level of the ventricles in a case of sudden death in a male patient, who was in his mid 20's, and found dead in the street in the early hours of the morning. There is intense left ventricular hypertrophy particularly affecting the interventricular septum (approximately 4·5 cm) with associated fibrosis and virtual obliteration of the left and right ventricular cavities.

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