Heart antibodies in cardiomyopathies

T TRUEMAN, R A THOMPSON, P CUMMINS, W A LITTLER

From the Department of Cardiovascular Medicine, University of Birmingham and Regional Immunology Laboratory, East Birmingham Hospital

SUMMARY The reported frequency of circulating heart reactive antibodies in cardiomyopathies has varied and their significance is unknown. In this study such antibodies were sought in patients with primary congestive and hypertrophic cardiomyopathies and other heart diseases.

Standard "single sandwich" and the more sensitive "double sandwich" indirect immunofluorescence techniques failed to disclose a significant difference between any cardiomyopathic group and controls in repeated experiments.

With both techniques results were subject to considerable method-specific artefacts and observer variation. No published work associating heart antibodies detected by immunofluorescence methods with cardiomyopathies adequately takes these into account.

Circulating heart antibodies detected by immunofluorescence techniques in rheumatic fever and post-myocardial injury syndromes may be of pathogenic significance.^{1 2} Their presence in ischaemic heart disease^{3 4} and in the post-cardiotomy state⁵ presumably merely reflects cardiac damage, while their presence in other disorders^{6 7} suggests they may be a non-specific finding.

Heart antibodies have been detected in patients with congestive and hypertrophic cardiomyopathies by some investigators⁸⁻¹² but not by others.^{3-5 13 14} Their significance is unknown. They may continue to damage myocardium after insults such as viral infection.¹⁵ Evidence that cardiomyopathy may follow myocarditis, however, is circumstantial and anecdotal.¹⁶

We report here the experience of this department in the detection by immunofluorescence methods of heart antibodies in cardiomyopathies.

Subjects and methods

(1) SUBJECTS

Of 86 patients, 13 had idiopathic congestive and 11 hypertrophic cardiomyopathy, according to the classification of Goodwin,¹⁷ the diagnosis being confirmed by cardiac catheterisation in nine of the former and 10 of the latter. Seven patients had myocarditis and eight had ischaemic heart disease. Twelve patients had congestive cardiomyopathy and habitually consumed more than 650 g alcohol per week (alcoholic cardiomyopathy group). Eight had congestive cardiomyopathy in association with

diastolic pressures consistently greater than 110 mmHg (hypertensive cardiomyopathy group). The post-infarct/cardiectomy group consisted of five patients with possible post-myocardial infarction syndrome and six with recent or remote cardiac surgery but no evidence of post-cardiotomy syndrome. Sixteen patients had other miscellaneous cardiovascular disorders (Table 1). Patients were classified functionally according to New York Heart Association criteria with Class II subdivided into IIa (symptoms only on fairly heavy exertion) and IIb (symptoms on mild exertion, for example walking on the flat). The presence or a history of congestive heart failure and the cardiothoracic ratio on a standard posteroanterior chest radiograph were noted. Forty healthy volunteers were control subjects.

Table 1 Diagnoses of patients in miscellaneous (MISC) group

	No.
'Idiopathic' pericarditis	3
Tuberculous pericarditis	1
Constrictive pericarditis	1
Complete heart block	2
Thyrotoxic heart disease	1
Anomalous pulmonary venous return	
and constrictive pericarditis	1
Left atrial myxoma	1
Right atrial thymoma	1
Angina with normal coronary arteries	1
Thalassaemia major with decreased	
left ventricular compliance	1
Mitral valve prolapse	1
Bradyarrhythmias	1
Hypertension	1

Received for publication 12 December 1980

Sera were separated from cells immediately, divided into aliquots, and stored at -35°C or below. Sera (kindly supplied by Professors Goodwin and Waterson) from eight patients with idiopathic congestive cardiomyopathy attending the Hammersmith Hospital, were kept at -20°C before transfer to Birmingham. Sera from nine patients with recurrent pericarditis (kindly supplied by Professor Mowbray at St Mary's Hospital, London) had spent one or two days on the cells at ambient temperatures before separation and storage at -20°C . Both groups of sera were transferred to Birmingham in solid carbon dioxide and stored at -35°C or below.

(2) IMMUNOFLUORESCENCE

Pilot experiments were performed in which dilutions of sera and reagents, and incubation and washing times were varied. The methods described below are those that distinguished weakly positive from negative reactions in an optimal way in this laboratory.

Substrate

Normal baboon left ventricle, taken immediately after death, was frozen in isopentane (cooled in liquid nitrogen) and stored at -70°C . Six micron sections were cut on a cryostat at -20°C and air dried on microscope slides for 15 minutes. Though any mammalian myocardium might have been used^{8 18-20} the baboon's was chosen as primate heart may share antigens with human heart not found in other mammals²¹ and fresh material was available. Non-myopathic human left ventricle obtained less than five hours after death gave identical results to those obtained using material from the baboon.

Reagents

Phosphate buffered saline (PBS)—0.15 M, pH 7.2
Fluorescein labelled sheep anti-human immunoglobulin (Wellcome Reagents Ltd., Beckenham)

Sheep anti-human IgG, Fc fragment (SERA-Lab Ltd., Crawley Down)

FITC-labelled rabbit anti-sheep IgG (Regional Immunology Laboratory, East Birmingham Hospital)
F. A. rhodamine counterstain 0.5% in PBS (Difco Laboratories, Detroit)

Phosphate buffered glycerol—9 parts glycerol, 1 part of PBS 10X concentrate

Standard ('single sandwich') technique

Sera were diluted 1/5 with phosphate buffered saline and 0.1 ml solution applied to tissue sections. These were incubated at room temperature in a moist chamber for 20 minutes. Slides were then washed. All washing was done for two consecutive 15 minute periods in fresh phosphate buffered saline. Each section was then covered with one drop

(approximately 0.03 ml) of a 1/25 solution of fluorescein labelled sheep antihuman immunoglobulin in rhodamine solution and incubated for 15 minutes. After being washed again the sections were mounted in phosphate buffered glycerol and examined under the ultraviolet microscope. Known positive sera and phosphate buffered saline were used as controls in each experiment.

Modified ('double sandwich') technique

After 30 minutes of incubation with diluted sera tissue sections were washed and incubated with 1/80 sheep anti-human IgG, Fc in phosphate buffered saline for 30 minutes. They were washed and incubated with a 1/5 solution of FITC-labelled rabbit anti-sheep IgG in rhodamine for 15 minutes, washed again, and mounted in buffered glycerol. In addition to those controls used in the single sandwich experiments, phosphate buffered saline was used instead of sheep antihuman IgG, Fc on sections treated with a strongly positive serum. No fluorescence was seen in sections so treated.

Microscopy

A Vickers M41 epi-illumination microscope with HBO 200 watt mercury vapour lamp was used, with Balzer interference excitor filters and OG1 yellow and BG 38 barrier filters. Sections were read independently by two observers (TT and RAT). Intensity of fluorescence was graded on a scale 0 to + + + +. A serum was said to be positive when both observers recorded fluorescence of + or more intensity. If one observer rated fluorescence as greater than + intensity it was recorded as such. Fluorescent staining patterns were classified according to Nicholson and co-workers.²²

(3) EXPERIMENTAL DESIGN

Groups were compared with respect to the proportion exhibiting '+' and '>+' fluorescence in repeated single and double sandwich tests. Except in one single sandwich experiment, comparing congestive and alcoholic cardiomyopathy sera with controls, all tests were read blind, neither observer knowing which sections had been treated with which sera.

Enough sera were tested by the double sandwich method to compare groups classified according to NYHA criteria, history of congestive failure, and cardiothoracic ratio. It was also possible to match each member of the congestive cardiomyopathy group for age and sex with a control and a member of the miscellaneous group.

Comparisons of the proportions of positive results in patient groups were made using the standard error of difference of proportions and χ^2 technique incorporating the correction factor of Yates.

Table 2 Experiment 1: single sandwich

Patient group	No. tested	No. positive	Significance of difference from controls
Congestive cardiomyopathy	8	3	p<0.05
Alcoholic cardiomyopathy	6	0	NS
Control	27	0	

Table 3 Experiment 2: single sandwich

Patient group	No. tested	No. positive	Significance of difference from controls
Congestive cardiomyopathy	9	4	NS
Alcoholic cardiomyopathy	9	4	NS
Hypertensive cardiomyopathy	6	1	NS
Hypertrophic cardiomyopathy	9	1	NS
Myocarditis	6	2	NS
Post-infarct cardiomyopathy	9	6	p<0.02
Miscellaneous and ischaemic heart disease	16	2	NS
Control	30	6	

Results

(1) SINGLE SANDWICH

The results of the non blind study are shown in Table 2. The apparently significant excess of heart antibody activity in the congestive cardiomyopathy group was not seen when a greater number of sera were read blind (Table 3). Only in the post-infarct/cardiomyopathy group was there a significantly greater proportion with positive results compared with controls. Fluorescence of more than '+' intensity was virtually limited to this group (4/9 compared to 1/30 control, p<0.02).

In five further experiments on selected patient groups none differed significantly from controls. In one experiment a significantly greater proportion of locally obtained congestive cardiomyopathy sera showed fluorescence than did such sera obtained from the Hammersmith Hospital.

Only post-infarct/cardiomyopathy sera, used in each experiment as controls, were consistently read as positive by both observers. The fluorescence exhibited by other subjects was much weaker and results varied between experiments. The results of single observers also differed between experiments. For instance, in experiments three and four only 50% of sera were interpreted the same (that is positive or negative) by RAT.

Agreement (positive versus negative) between observers was present in 74% of sections viewed. Only 59.7% of those found positive by TT were read as positive by RAT and 60.7% vice versa.

PATTERNS OF FLUORESCENCE

Separate analysis of results according to the pattern of fluorescence showed no consistent difference between groups. The following patterns were observed.

Striated muscle specific

Peripheral: this pattern, described as subsarcolemmal-sarcoplasmic by some authors and possibly that described as sarcolemmal-subsarcolemmal by others, is the most specific for striated muscle.²² It describes a progressive increase in fluorescence from the centre to the periphery of the myofibre. When only of '+' intensity it was often difficult to distinguish from diffuse staining. Occasionally both patterns were present. Clear strong peripheral staining was seen only in members of the post-infarct/cardiomyopathy group.

Diffuse: in the absence of other staining most sera exhibited weak, even staining of myofibres making distinction between '0' and '+' intensity diffuse fluorescence difficult.

Striational: strong striational staining was observed in sections with a peripheral pattern. Finer striational fluorescence was seen in sections staining diffusely.

Non-specific

Intercalated disc: this pattern was only apparent in the absence of other more specific staining patterns and its presence depended on the orientation of muscle fibres within the section.

Endomyseal: fluorescence of connective tissue between bundles of myofibres was seen in all sections without strong peripheral or diffuse staining. It was seen in sections incubated with phosphate buffered saline instead of serum. Intense endomyseal fluorescence was exhibited by two sera, from a patient with probable post-myocardial infarction syndrome and from a patient with a thymoma invading the right atrium.

Intermyofibrillar: faint linear staining between myofibrils, parallel to their long axes, was seen only in some sections without significant staining patterns.

Nuclear: two control and one post-infarct/cardiomyopathy serum exhibited antinuclear antibody.

There was considerable interexperimental variation in the patterns recorded. In experiment 4 nine of the 16 positive reactions were peripheral but in experiment 7, 16 of the 17 positive reactions were diffuse. In contrast, the strong fluorescence of post-infarct/cardiomyopathy sera was consistently of a peripheral pattern with well marked striations.

(2) DOUBLE SANDWICH

No cardiomyopathy group showed fluorescence in a significantly greater proportion than controls though sera from other centres exhibited fluorescence

Table 4 Double sandwich

Patient group	No. tested	No. of positive tests		Significance of excess (+) or deficiency (-) over expectation from controls	
		+ or more	more than +	+ or more	more than +
Congestive cardiomyopathy	13	7	5	+NS	+NS
Alcoholic cardiomyopathy	12	6	2	+NS	+NS
Hypertensive cardiomyopathy	8	2	1	-NS	—
Hypertrophic cardiomyopathy	11	4	1	-NS	-NS
Myocarditis	7	3	3	+NS	+NS
Ischaemic heart disease	8	6	2	+p<0.05	+NS
Miscellaneous	16	8	3	+NS	+NS
Post-infarct cardiomyopathy	11	7	6	+p<0.05	+p<0.02
Recurrent pericarditis	9	0	0	-p<0.02	-NS
Hammersmith congestive cardiomyopathy	8	1	0	-p<0.05	-NS
Control	40	16	5		

significantly less often than locally obtained congestive cardiomyopathy sera (Table 4). Recurrent pericarditis sera did so significantly less often than controls. Only in the ischaemic heart disease group was a significantly greater proportion positive than in controls.

Only the post-infarct/cardiomyopathy group exhibited '>+' fluorescence in a significantly greater proportion than controls. Sera from other centres showed such fluorescence significantly less often than locally obtained congestive cardiomyopathy sera.

No significant differences were seen between the congestive cardiomyopathy group and age and sex matched members of the miscellaneous group and controls.

Intensity of staining was greater in the post-infarct/cardiomyopathy group than in any other. In repeated experiments consistent results were observed only in this group.

Positive fluorescence appeared to be related to

severity of disease within congestive, alcoholic, and hypertensive cardiomyopathy groups (Table 5). A statistically significant difference was observed between the proportions positive of those patients divided on the basis of NYHA classification.

The double sandwich method was more sensitive than the single sandwich technique. Dilutions of strongly positive sera from asymptomatic patients after mitral valvotomy were distinguishable from appropriately diluted controls to a titre of 1/160 with this method and 1/40 with the single sandwich technique when both were performed simultaneously.

PATTERNS OF FLUORESCENCE

With this technique all sera, including controls, showed diffuse, striational, endomyseal, and intercalated disc staining to moderate intensity. Unless very intense, this fluorescence was ignored. No serum exhibited diffuse fluorescence significantly more intensely than others. Endomyseal and intermyofibrillar patterns were more apparent in the absence of other fluorescence. Clearly positive, reproducible, peripheral staining was seen almost exclusively in members of the post-infarct/cardiomyopathy group. Peripheral staining was usually accompanied by fairly strong striational fluorescence and a mottled intramyofibre pattern believed to represent such 'banding' on transverse section. Only with one serum, from a patient with probable post-infarction syndrome, was an unrelated staining pattern thought to be positive. Strong endomyseal fluorescence was shown by this serum, as in single sandwich studies. Antinuclear antibody was detected in the same sera as in single sandwich experiments.

Discussion

When sera were assessed blind no significant

Table 5 Experiment 8: double sandwich: proportions of congestive, alcoholic, and hypertensive cardiomyopathy groups with positive results

Patient group	No. tested	No. positive
NYHA Classification		
I, IIa	15	4
NYHA Classification		
IIb, III, IV	18	11 (excess significance p<0.05)
Congestive failure	23	12
No congestive failure	10	3
Cardiothoracic ratio		
≤0.54	14	4
>0.54	13	8

Table 6 Published immunofluorescence results in non-tropical cardiomyopathies

	Substrate	Congestive cardiomyopathy	Hypertrophic cardiomyopathy	Control	Other conditions
Fletcher and Wenger ¹⁴	Human	9/34	—	—	15/71 non-cardiac diseases
Camp <i>et al.</i> ¹³	Human	3/28	—	1/25	4/12 miscellaneous cardiovascular disease
Kirsner <i>et al.</i> ⁹	Human	9/32	—	5%	—
Robinson <i>et al.</i> ¹⁰	Human	8/50	—	0/40	—
Bauer <i>et al.</i> ⁴	Human	0/6	—	0/15	12/56 angina
Zabriskie <i>et al.</i> ⁵	Human	0/7	—	—	12/12 post-cardiotomy state
Bailey <i>et al.</i> ³	Human	8/17	—	—	5/14 ischaemic heart disease
Klajman <i>et al.</i> ¹⁸	Human and rabbit	Familial cardiomyopathy	14/28	2/36	—
Bachman <i>et al.</i> ¹⁹	Rat, guinea-pig, ox, rabbit, pig and Human	—	2/4	5/120	20% ischaemic heart disease
Das <i>et al.</i> ²³	Human	7/13	4/5	1/50	—
Das <i>et al.</i> ¹¹	Human	6/35	9/11	1/50	0/17 ischaemic heart disease
Oevermann <i>et al.</i> ²⁴	Rat, guinea pig, rabbit, and human	10/24	—	1/30	3/30 miscellaneous diseases
Bölte ⁸	Rat, guinea pig, and human	10/24	0/21	1/30	3/30 miscellaneous diseases
					0/18 alcoholic cardiomyopathy
Sack <i>et al.</i> ¹²	Human	35/42	25/25	0/31	0/33 recent myocardial infarction

difference was seen between healthy control subjects and patients with cardiomyopathies in terms of the presence, intensity, or pattern of fluorescence. When sera were not read blind an apparently significant difference was seen.

Among those patients with congestive forms of cardiomyopathy it appears that weak fluorescence reflected cardiac damage. This is supported by the finding of a significantly greater proportion showing '+' fluorescence in double sandwich experiments from patients with ischaemic heart disease than from controls.

The necessity for blind assessment in test sera alongside controls, the variation in interpretation of weak fluorescence, and the effect of minor variations in handling sera on such fluorescence led to a critical examination of reports relating positive immunofluorescence tests to cardiomyopathies (Table 6).

In no report associating heart antibodies with cardiomyopathies is it possible to ascertain if control sera were read blind with test sera. In one study, multiple samples were taken from patients but not control subjects.⁹ When single tests were performed on most of these patients a negative result was seen.¹³

Only a selection of the publications of Das and Bölte and their colleagues are listed.^{8 11 23 24} Their control groups remain the same despite the addition

of patients or patient groups. Inconsistent results of congestive cardiomyopathy sera are found by Das *et al.*^{11 23} Unlike Das, Bölte⁸ found no hypertrophic cardiomyopathy sera with positive results. He also found negative results in patients with alcoholic cardiomyopathy, a finding in disagreement with other reports.^{9 14} Eight of Bölte's 10 positive congestive cardiomyopathy sera exhibited 'sarcolemmal' fluorescence, but photographic representation of such fluorescence (personal observation, meeting of International Society and Federation of Cardiology, London, January 1978) showed only an endomyseal staining pattern which is of no significance.²² The report of Sack *et al.*¹² using a double sandwich technique shows a surprising lack of positive results in controls though this method is more sensitive and less specific than the single sandwich technique. Again no mention of how control and test sera were assessed is made.

It is concluded that circulating heart antibodies, as detected by immunofluorescence methods, are not present to a significant extent in patients with primary cardiomyopathies. If present at all they are non-specific and merely reflect cardiac damage.

We are grateful to Professors Goodwin, Waterson,

and Mowbray who supplied sera from their patients. TT was in receipt of a British Heart Foundation Junior Research Fellowship.

References

- Kaplan MH. Autoantibodies to heart tissue in the sera of certain patients with rheumatic fever (abstract). *Fed Proc* 1959; **18**: 576.
- van der Geld H. Anti-heart antibodies in the postpericardiectomy and the post myocardial-infarction syndromes. *Lancet* 1964; **II**: 617-21.
- Bailey JP Jr, Cundey PE Jr, Bush WE. Prevalence of myocardial antibodies (abstract). *Clin Res* 1968; **16**: 25.
- Bauer H, Waters TJ, Talano JV. Antimyocardial antibodies in patients with coronary heart disease. *Am Heart J* 1972; **83**: 612-9.
- Zabriskie JB, Hsu KC, Seegal BC. Heart-reactive antibody associated with rheumatic fever: characterization and diagnostic significance. *Clin Exp Immunol* 1970; **7**: 147-59.
- Shaper AG, Kaplan MH, Foster WD, Macintosh DM, Wilks NE. Immunological studies in endomyocardial fibrosis and other forms of heart disease in the tropics. *Lancet* 1967; **i**: 598-600.
- Das SK, Cassidy JT. Antiheart antibodies in patients with systemic lupus erythematosus. *Am J Med Sci* 1973; **265**: 275-80.
- Bölte H-D. In: Kaltenbach M, Loogen F, Olsen EGJ, eds. *Cardiomyopathy and myocardial biopsy*. Berlin, Heidelberg and New York: Springer-Verlag, 1978: 251-5.
- Kirsner AB, Hess EV, Fowler NO. Immunologic findings in idiopathic cardiomyopathy: a prospective serial study. *Am Heart J* 1973; **86**: 625-30.
- Robinson JA, Griebble HG, Anderson TO. The serological abnormalities of idiopathic myocardial disease. *Cardiovasc Res* 1970; **4**: 14-22.
- Das SK, Cassidy JT, Dodson VN, Willis PW III. Antiheart antibody in idiopathic hypertrophic subaortic stenosis. *Br Heart J* 1973; **35**: 965-9.
- Sack W, Sebening H, Wachsmuth ED. Auto-Antikörper gegen Herzmuskelsarkolemm im Serum von Patienten mit primärer Cardiomyopathie. *Klin Wochenschr* 1975; **53**: 103-10.
- Camp TF, Hess EV, Conway G, Fowler NO. Immunologic findings in idiopathic cardiomyopathy. *Am Heart J* 1969; **77**: 610-8.
- Fletcher GF, Wenger NK. Autoimmune studies in patients with primary myocardial disease. *Circulation* 1968; **37**: 1032-5.
- Wilson FM, Miranda QR, Chason JL, Lerner AM. Residual pathologic changes following murine Coxsackie A and B myocarditis. *Am J Pathol* 1969; **55**: 253-65.
- Anonymous. Virus, immunology and the heart (Editorial). *Lancet* 1979; **ii**: 1111-3.
- Goodwin JF. Clarification of the cardiomyopathies. *Mod Concepts Cardiovasc Dis* 1972; **41**: 41-6.
- Klajman A, Kamin-Belsky N, Feldman S, Kariv I. Immunologic studies in familial cardiomyopathy. *Am J Cardiol* 1971; **28**: 707-11.
- Bachmann GW, Galm Y, Rapp W. Der diagnostische Beitrag immunologischer Untersuchungsmethoden in der Kardiologie. 1. Darstellung zirkulierender Herzmuskelantikörper mit der indirekten Immunfluoreszenz. *Widerbelebung und Organersatz* (suppl to *Z Kreislaufforsch*) 1967; **4**: 119-25.
- Gaul G, Ludwig H, Steffen C. Vergleichende Untersuchung über das Vorkommen herzreaktiver Antikörper als Aktivitätsnachweis bei rheumatischen Herzerkrankungen mittels Humanherz- und Affenherzantigen im Konsumtionstest und Immunfluoreszenztest. *Wien Klin Wochenschr* 1974; **86**: 532-9.
- Espinosa E, Kaplan MH. Antigenic analysis of human heart tissue. Antigens with restricted organ distribution in acid extracts of human myocardium. *J Immunol* 1970; **105**: 416-25.
- Nicholson GC, Dawkins RL, McDonald BL, Wetherall JD. A classification of anti-heart antibodies: differentiation between heart-specific and heterophile antibodies. *Clin Immunol Immunopathol* 1977; **7**: 349-63.
- Das SK, Dodson VN, Willis PW. Antiheart immunoglobulins in cardiomyopathy, especially in hypertrophic muscular subaortic stenosis (abstract). *Am J Cardiol* 1969; **23**: 108-9.
- Oevermann W, Bölte HD, Zwehl W. Indirekter Immunfluoreszenztest und indirekter Antiglobulinkonsumtionstest in der Diagnostik primärer Kardiomyopathien. *Verh Dtsch Ges Inn Med* 1973; **79**: 1121-4.

Requests for reprints to Professor W A Littler, Department of Cardiovascular Medicine, East Birmingham Hospital, Bordesley Green East, Birmingham B9 5ST.