Humoral immunity in cardiomyopathy

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SUMMARY Sera from patients with heart disease were examined by single sandwich indirect immunofluorescence for the presence of antibodies to human heart muscle. Endocardial biopsy specimens were also examined by direct immunofluorescence for deposition of antibodies *in vivo*. No consistent or specific abnormality was found in the biopsy specimens or sera of patients with congestive cardiomyopathy. The presence of anti-heart antibodies in cardiac disease appears to reflect damage to cardiac muscle whatever the cause. Immunofluorescence is an insensitive test for anti-heart antibodies.

It has been suggested that cardiac damage in congestive cardiomyopathy may be mediated by an autoimmune mechanism.¹ The role of humoral abnormalities, in particular type II hypersensitivity, has received considerable attention,²⁻⁴ but the results and their interpretation vary widely. Previous findings in our department (using baboon heart as substrate) suggested that the role of humoral autoimmunity as a primary cause of congestive cardiomyopathy is not proven⁵; we undertook a further study to confirm or refute the findings.

Our aims were (a) to detect serum autoantibodies to human heart muscle, (b) to detect evidence of *in vivo* antibody fixation by cardiac antigens, and (c) to demonstrate immune complex deposition in and around myocardial vessels.

Patients and methods

SEROLOGICAL STUDY

Sera of 95 patients were examined for the presence of antiheart antibodies. Forty three had cardiomyopathy according to the classification of Goodwin and Oakley⁶: 28 had congestive cardiomyopathy diagnosed both clinically and by cardiac catheterisation and endomyocardial biopsy, with one exception in which the diagnosis was confirmed at necropsy and 15 had hypertrophic cardiomyopathy, in all but one of whom the diagnosis was confirmed at catheterisation. Nine of the 15 patients in the latter group had a left ventricular outflow tract obstruction detected at catheter-

isation. Sixteen patients had ischaemic heart disease and nine rheumatic heart disease; six subjects were healthy controls; and the remaining 21 patients comprised a miscellaneous group including five with post-infarct/post-cardiotomy syndrome (Table 1). The groups were classified symptomatically according to the New York Heart Association (NYHA) criteria at the time of venesection (Table 2): 50% of patients with congestive cardiomyopathy and 44% with ischaemic heart disease were in groups IIb, III, and IV.

Serum was separated from cells immediately after venesection and stored at -20°C. Sections from normal human left ventricle and congestive cardiomyopathic left ventricle were used as the substrate. The normal heart was obtained within 14 hours of death from a woman who died after trauma. The congestive cardiomyopathic heart was obtained within 24 hours of death from a 16 year old boy in whom the diagnosis of congestive cardiomyopathy had been confirmed by invasive investigation and necropsy. The hearts were stored at -70°C. Sections of 7 µm were cut on a cryostat at -20°C from portions of these ventricles and dried in air for at least 10 minutes before storage at -70°C until use.

The single sandwich immunofluorescence technique was used, as previous work in this department⁵ has not shown any appreciable advantage of the double sandwich technique. Sera were tested at a dilution of 1/5 in phosphate buffered saline (PBS): 0.5 ml was incubated with sections of normal and congestive cardiomyopathic heart at room temperature for 30 minutes. The serum was removed by washing with PBS for five minutes. A drop (approximately 0.03 ml) of fluorescein isothiocyanate conjugated anti-human

Table 1 Diagnoses in miscellaneous group of 21 patients with heart disease

Diagnosis	No. of patients examined by:	
	Serology	Biopsy
Post-infarct/post-cardiotomy syndrome	5	
? Cardiomyopathy	5	2
Pericarditis or myocarditis or both	4	3
Hypertension	3	2
Constrictive pericarditis	1	1
Post-heart transplant (for CCM)	1	
Systemic lupus erythematosus	1	
Non-rheumatic mitral regurgitation	1	
Rheumatic heart disease		1
Aortic incompetence		1
Discrete subaortic membrane		1
Normal		1

CCM, congestive cardiomyopathy.

IgG (Atlantic Antibodies) was placed on each section, and after a further 30 minutes the slides were washed in PBS, mounted in buffered glycerol, and viewed under the fluorescence microscope (Leitz Ortholux). A negative control serum sample from a healthy subject and a known positive serum sample were included in each batch of slides. The sections were viewed blindly and independently by two observers. Fluorescence was graded as positive (+); weak or no fluorescence was regarded as negative (-). Each patient had serum viewed on two sections, one congestive cardiomyopathic heart and one normal heart: thus if both observers graded fluorescence as positive in both sections a maximum total of 4+ could be scored for the patient.

BIOPSY STUDY

Fifty four endomyocardial biopsy specimens were obtained at the time of cardiac catheterisation from 53 subjects. Of these, 22 had congestive cardiomyopathy, 14 hypertrophic cardiomyopathy, and five ischaemic heart disease. The diagnoses in the

Table 2 Groups classified according to New York Heart Association criteria

Classification group:	0	I	IIa	IIb	III	IV
Congestive cardiomyopathy (n=28)	1	5	8	6	5	3
Ischaemic heart disease (n=16)	2	3	4	3	3	1
Hypertrophic cardiomyopathy (n=15)	7	0	4	1	2	1
Rheumatic heart disease (n=9)	1	1	2	3	1	1
Miscellaneous (n=21)	13	1	3	2	1	1

remaining 12 are given in Table 1. Thirteen of the first group and six of the second were also included in the serological study for anti-heart antibodies.

The biopsy specimens were obtained at cardiac catheterisation using a King's biptome⁷ from the right ventricular septum in three cases and from the left ventricle in the remainder. Each specimen was placed in a screw topped ampoule, flash frozen in liquid nitrogen within 30 minutes of collection, and stored at -70°C. The specimens were mounted in OCT embedding compound (Miles Laboratories) and eight 7 µm sections were cut on to a slide at -20°C in a cryostat, air dried for at least 10 minutes, and stored at -70°C. When required, the sections were air dried and washed in PBS for 10 minutes. The conjugates were placed on the slides: fluorescein isothiocyanate conjugated anti-human IgG, IgM, and IgA (Atlantic Antibodies), complement (C3) (Kallestad), and fibrin (Hyland) were used. The sections were incubated for 30 minutes in a moist box at room temperature, rinsed, and washed in PBS for 10 minutes. The sections were mounted in 90% glycerol and viewed under the ultraviolet microscope. The sections were viewed blindly and independently by two observers (PJJ and RT). The presence and site of increased fluorescence were noted.

All sections were viewed under a Leitz Ortholux II microscope with Epi illumination using a super pressure mercury source (HBO 50), a Balzer filter exciter, a red cut off filter, and a yellow barrier filter.

STATISTICAL METHOD

The significance of the intraobserver variation was measured using the χ^2 test. Comparisons between the patient groups were made using the χ^2 test for independent samples.⁸

Results

SEROLOGICAL STUDY

All 95 patients had sera examined: 16 had the test repeated on at least one occasion using the same stored sera. The observers' findings were in agreement for 73% of the sections viewed. In those cases in which the test was repeated, each observer agreed with the former assessment in 66% (PJJ) and 62% (RT): this intraobserver error was significant (0.05 > p > 0.02). On two occasions the negative control was deemed positive, and on seven of 16 (44%) observations the positive control was classed as negative.

Observations with congestive cardiomyopathic heart as substrate were in agreement more often (85% compared with 61%) than with normal heart substrate. Repeat tests were also in agreement more often with cardiomyopathic heart (71% compared with

Table 3 Distribution of positive and negative fluorescence in 95 patients with heart disease

Diagnostic group	Total No. of patients	Fluorescence results					Total No. (%) positive observations
		0	1+	2+	3+	4+	
Congestive cardiomyopathy	28	14	8	3	3	0	24/116 (21)
Hypertrophic cardiomyopathy	15	7	5	2	1	0	15/76 (20)
Ischaemic heart disease	16	6	5	2	3	0	20/74 (27)
Rheumatic heart disease	9	4	3	2	0	0	8/38 (21)
Miscellaneous	16	9	6	1	0	0	8/70 (11)

Table 4 Number of biopsy specimens with increased fluorescence in identified sites. (Figures in parentheses are number weakly affected)

	Congestive cardiomyopathy (n=22)					Hypertrophic cardiomyopathy (n=14)					Ischaemic heart disease (n=5)				
	Fibrin	IgG	C3	IgM	IgA	Fibrin	IgG	C3	IgM	IgA	Fibrin	IgG	C3	IgM	IgA
Fibrous tissue	8	2				3	1				1				
Endothelium	2	3	1	1		1			1	1					
Subendothelium	3	3				2	2	1	(1)		2				
Perimyoeal	6	7	1(1)	1(1)	1	2	2								
Intermuscular septa	3	2		1	2	1					1	(1)			
Degenerating muscle	3	3	1	1	1		(1)								
Muscle bundles	1	3(1)		1(1)	1	1	1								
Capillaries			1(2)	1				1					(1)	(1)	(1)

44%) than with normal heart, but the positive control was classed as negative on 50% of the observations with cardiomyopathic heart compared with 38% with normal heart substrate.

Table 3 shows the distribution of positive and nega-

Table 5 Results of fluorescence in serological and biopsy studies in patients with congestive and hypertrophic cardiomyopathies

No. of patients	Serology (fluorescence)	Biopsy (fluorescence with conjugates)
Congestive cardiomyopathy		
5	-	Fibrin in connective tissue
8	-	Slight C3 and IgM in capillaries, fibrin in connective tissue
43	-	IgG and fibrin in some myofibres
37	-	Fibrin in fibrous tissue
26	-	Fibrin in fibrous tissue, some IgG and IgM between muscle bundles
4	-	
41	+	
13	+	
51	+	Fibrin and IgG in subendothelium and connective tissue
2	+	Fibrin and IgG in subendothelium and connective tissue
50	+	Fibrin and IgG in subendothelium and connective tissue, area of degenerating muscle stains for all
7	++	
46	+	IgG and fibrin in septa, IgG in some muscle, weak IgM and IgA in septa
Hypertrophic cardiomyopathy		
10	-	C3, IgG, and fibrin in subendothelium, C3 in capillaries
18	-	
20	-	IgG and fibrin in connective tissue
14	+	
48	++	
36	+++	

Fluorescence: -, negative; +, ++, +++, positive.

tive fluorescence results in the different groups. There was no significant difference in the number of sera showing fluorescence on at least one observation ($0.90 > p > 0.08$). Sera from only one normal subject gave a positive result, and all those from the post-infarct/post cardiectomy group showed fluorescence on at least one occasion. There was no significant difference in the total number and percentage of positive observations within each group (Table 3). The percentage of positive observations in the miscellaneous control group was 8% and for the post-infarct/post-cardiectomy group 50%. When the symptomatic (NYHA) groups O-IIa with IIb-IV are compared positive fluorescence was seen on at least one occasion in 53% of sera from the milder group and in 59% of those from the more severe group (not significant); and the percentage of positive observations in the milder group was 22% and 23% in the more severe group (not significant).

BIOPSY STUDY

Table 4 shows the results of fluorescence on the biopsy specimens. In the hypertrophic group one specimen was of poor quality and unsuitable for interpretation. In the miscellaneous group fibrin and IgG were increased in areas of fibrosis in the cases of constrictive pericarditis, hypertension, and discrete subaortic stenosis. One patient with myocarditis had increased fibrin and IgG, C3, IgM, and IgA fluorescence in the thickened endocardium and IgG and fibrin in the connective tissue. Fibrin and IgG were bound in the septa of the one normal subject.

Table 5 shows the serological and biopsy results in patients included in both parts of the study with con-

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gestive cardiomyopathy and hypertrophic cardiomyopathy. Six of 13 patients with congestive cardiomyopathy, three of six patients with hypertrophic cardiomyopathy and five of the 12 in the miscellaneous group had some degree of immunofluorescence in the serum. In the first group, absence of positive fluorescence in the serum did not always infer binding of antibody in the biopsy specimen and often fluorescence was seen in both sera and biopsy specimens.

Discussion

The specificity of the immunofluorescence test for anti-heart antibodies in serum was 93% but the sensitivity was only 56%. The test is therefore specific for these antibodies as few false positives occur, and this is borne out in the control group, but the percentage of false negatives is so high that results cannot give a true reflection of the incidence of anti-heart antibodies. Our results show that the antibodies appear to be present to some extent in all forms of heart disease, that they do not differ significantly between groups, and that anti-heart antibodies probably reflect non-specific cardiac damage. This is in agreement with other studies.^{3,9} Although sera from all the patients in the post-infarct/post-cardiotomy group showed fluorescence on at least one occasion, only 50% of all the observations in this group were positive, reflecting the low sensitivity of the test. As reported elsewhere¹⁰ the results for the patients with more severe symptoms did not differ significantly either within their groups or between the groups and do not support the previous finding in our laboratory⁵ or Bolt and Grothey's findings.²

There were wide interobserver and intraobserver variations: only 73% of observations were in agreement, and on repeat testing the observer agreed with his former assessment on only 66% (PJL) and 62% (RT) of occasions ($0.05 > p > 0.02$). Assessment of fluorescence is subjective and there is no accepted method of quantifying the amount of fluorescence. It is important that tests are performed blind and with more than one observer, otherwise inconsistencies are bound to arise.^{11,12}

In our previous study⁵ fresh baboon heart was used as substrate; in the present study fresh normal human heart and fresh cardiomyopathic human heart were used and no difference in results was obtained.

There was no consistent pattern of fluorescence in any group: the sites of fluorescence and the proportion of biopsy specimens showing fluorescence did not differ between the groups. Increased fibrin staining usually reflected fibrosis regardless of the aetiology and is therefore secondary to damage. Increased fluorescence with C3 was rarely seen, and then in no consistent site, suggesting that complement mediated

damage does not occur in congestive (or hypertrophic) cardiomyopathy. IgG was seen in apparently normal muscle bundles in only three patients (slightly in one other) in the congestive cardiomyopathy group and in one patient in the hypertrophic cardiomyopathy group; thus it can be concluded that the primary mechanism of muscle damage in congestive cardiomyopathy is not by autoantibodies with or without complement. In only occasional cases in all groups did fluorescence staining occur in the capillary walls; thus immune complexes do not appear to be the cause of tissue destruction. These findings confirm earlier work in our department¹³ and contrast with the results of other workers^{2,14} whose studies were not performed blind. We believe that binding of immunoglobulin is not specific to cardiomyopathy¹⁵ and certainly cannot be incriminated as the primary site of damage, as suggested by Sanders and Ritts.¹⁴ Das *et al.*¹⁶ postulated that anti-heart antibodies are not present in the serum because they are bound by antigen in the heart: the findings of the present study and former work¹³ do not support this view.

The immunofluorescence test, although specific for anti-heart antibodies, has low sensitivity and the true incidence of these antibodies in patients with congestive cardiomyopathy (or any other heart disease) cannot be assessed by this method. Our results do suggest that patients with this disease do not differ substantially from patients with other forms of heart disease when studied in this way. A primary role for humoral immunity in congestive cardiomyopathy cannot be entirely dismissed as the relevant antibody may not have been detected by this technique or the appropriate antigen may be inaccessible or absent in a cardiac biopsy specimen. We have, however, failed to prove an important role for humoral immunity in the pathogenesis of congestive cardiomyopathy. We have been unable to detect circulating antibodies specific to this disease or specific antigen in cardiomyopathic heart binding antibody, and in the biopsy specimens obtained no immune complex deposition was seen. The anti-heart antibodies detected by immunofluorescence probably reflect damage to cardiac muscle regardless of the cause.

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Notice

British Cardiac Society

The Autumn Meeting will be held at Wembley on 21 and 22 November 1983, and the closing date for abstracts was 26 July 1983.

The Annual General Meeting for 1984 will take place in Leicester on 11 and 12 April 1984, and the closing date for receipt of abstracts will be 3 January 1984.

The Autumn Meeting in 1984 will be held on 3 and 4 December 1984, and the closing date for receipt of abstracts will be 15 August 1984.