European Heart Journal (1997) **18**, 270–275

Circulating cardiac-specific autoantibodies as markers of autoimmunity in clinical and biopsy-proven myocarditis

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Aim Myocarditis and dilated cardiomyopathy may be phases of an organ-specific autoimmune disease of the myocardium. To provide evidence for autoimmune involvement in myocarditis, cardiac autoantibodies were detected in patient sera from the Myocarditis Treatment Trial.

Methods and Results Cardiac antibody status was assessed by indirect immunofluorescence and by anti-α-myosin enzyme-linked immunosorbent assay in 53 patients from the Myocarditis Treatment Trial (35 males, aged 42 ± 15 years); all had clinical myocarditis, but only 24 were classified as having histological myocarditis (Dallas criteria). By immunofluorescence, cardiac antibodies were more common in myocarditis (13/53) than in ischaemic (11/186, $P=0.0001$) or in normal controls (24/270, $P=0.001$). Abnormally raised anti-α-myosin antibodies were also more frequent in myocarditis (9/53) than in ischaemic (4/92, $P=0.01$) or normal controls (4/203, $P=0.0001$); 34% of myocarditis patients were positive with one or both tests. Similar proportions of patients with and without histological myocarditis had antibodies by immunofluorescence (8/24 vs 5/29, P=ns) and by enzyme-linked immunosorbent assay (4/24 vs 5/29, P=ns).

Conclusion The detection of disease-specific cardiac autoantibodies supports autoimmune involvement in a subset of patients with clinical myocarditis. The lack of correlation of antibody with biopsy features suggests that diagnosis of myocarditis should not be made on histology alone. Autoimmune markers may provide adjunct diagnostic tools and identify patients in whom immunosuppression is of potential benefit.
(Eur Heart J 1997; 18: 270–275)

Key Words: Myocarditis, autoimmunity, myosin, cardiac autoantibodies.

Introduction

Dilated cardiomyopathy is a chronic heart muscle disease of unknown cause[1–2]. The finding of autoantibodies to cardiac autoantigens in sera from patients with this condition suggests an immune pathogenesis[3–11]. Using indirect immunofluorescence or α-myosin specific enzyme-linked immunosorbent assay (ELISA), disease-specific cardiac autoantibodies are detected in 30% of patients with dilated cardiomyopathy at diagnosis[8,12]. The recent observation of these antibodies in 20% of their symptom-free relatives provides further evidence for autoimmune involvement[12,13].

Myocarditis seems to be the precursor of dilated cardiomyopathy in some cases, although diagnosis remains problematic[14]. The commonest cause of myocarditis is believed to be a virus infection, but the majority of cases remains of unknown aetiology[15–16]. Clinical and experimental features suggest that, at least in a patient subset, myocarditis and dilated cardiomyopathy might represent the acute and chronic stages of a progressive autoimmune disease of the myocardium[9,17–25]. The finding in patients with clinical and biopsy-proven myocarditis of the same autoantibody markers found in dilated cardiomyopathy[8,10,12,13] would be consistent with this hypothesis. To this end, we used indirect immunofluorescence and α-myosin specific ELISA to detect cardiac autoantibodies in sera from Myocarditis Treatment Trial patients[26].

Revision submitted 20 May 1996, and accepted 29 May 1996.

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0195-668X/97/020270+06 $18.00/0

Methods

Patients

The study population consisted of 53 patients (mean age 42 ± 15 years, 35 males) with clinical myocarditis from the Myocarditis Treatment Trial[26]. All underwent cardiac catheterization, coronary angiography and endomyocardial biopsy; at least four specimens were obtained from each patient for light microscopical evaluation. In 24 of the 53 patients, biopsy findings were consistent with myocarditis (Dallas criteria)[14]. The other 29 patients, in whom the Dallas criteria were not fulfilled, were not enrolled in the Trial. Patient sera were obtained from the Trial Investigators and tested blindly from diagnosis.

Cardiac antibodies by indirect immunofluorescence

Serum samples were tested by indirect immunofluorescence at 1/10 dilution on 4 μm-thick unfixed fresh frozen cryostat sections of blood group O normal human atrium and skeletal muscle, as described[8,13]. Cardiac antibody titres were measured by doubling dilutions of sera in phosphate-buffered saline solution. Cardiac antibody patterns were classified as reported[8,13]. Briefly, 'organ-specific' antibodies produced a diffuse cytoplasmic staining of myocytes; 'cross-reactive 1' antibodies gave a fine striational immunofluorescence on cardiac tissue, but stained only weakly skeletal muscle fibres; 'cross-reactive 2' antibodies stained with a striational pattern both heart and skeletal muscle sections. Absorption studies with relevant tissues had confirmed the organ-specificity and cross-reactivity of the three antibody types[8]. Two sera were used as standard positive (antibody titre 1/40) and negative controls and titrated in every assay. The intensity of immunofluorescence of the positive standard at 1/40 dilution was used as the threshold for positivity. All sera tested at 1/10 dilution were read blindly against these standards. An additional positive control serum was titrated to assess reproducibility. End point titres for this serum were reproducible within one double dilution in all assays.

The normal controls for the immunofluorescence test were 270 subjects (mean age 40 ± 11, 130 males) with normal clinical and non-invasive assessment; 186 patients (aged 52 ± 11 years, 130 males) with coronary artery disease, clinical heart failure and reduced angiographic left ventricular ejection fraction (mean 30 ± 8%) were also studied. All 186 patients underwent complete evaluation, including selective coronary angiography; 56 had had a myocardial infarct within 6 months prior to serum sampling.

Anti-α-myosin antibodies by ELISA

Atrial tissue, obtained from one normal donor heart at the time of transplantation, was frozen in liquid nitrogen and stored at −80° C until used. The α-(atrial specific)-myosin samples were prepared as described[10]. The ELISA for detection of anti-α-myosin antibodies has been previously detailed[12]. Briefly, ELISA plates (Immulon 1; Dynatech, West Sussex, U.K.) were coated with sequential duplicates of 100 μl purified human α-myosin at a concentration of 5 $\mu g \cdot ml^{-1}$. Sera were diluted at 1/320 in phosphate-buffered saline solution (Sigma, U.K.) containing 0·1% Tween 20 and 1% bovine serum albumin. Absorbance was assessed using a Pasteur Diagnostics ELISA reader at 450 nm. All antibody levels are expressed as mean absorbance at 450 nm ± standard error of the mean. The upper limit of normal for the assay was defined as 2 standard deviations (SD) above the mean value obtained from normals.

The normal control population for the ELISA included 203 individuals (aged 45 ± 16 years, 100 males) who had normal clinical and non-invasive assessment. The ischaemic heart disease control group included 92 patients (aged 63 ± 11, 65 males) with unstable angina; their clinical features have been described[12].

Statistical analysis

Quantitative data are given as means ± SD. Student's t-test, one-way analysis of variance, chi-square or Fisher's exact test were used as appropriate. All P values were two-tailed; statistical significance was defined as P values <0·05.

Results

Patient characteristics

Dallas positive and Dallas negative patients had similar age and sex distribution (Table 1). Among Dallas positive patients only one (4%) was in functional class I, six (25%) were in class II, 13 (54%) in class III and four (17%) in class IV. Among Dallas negative patients, four (14%) were in class III; haemodynamic data in these four patients are shown in Table 1. In the remaining 25 Dallas negative patients no data were available. Myocarditis was focal in 17 (71%) and diffuse in seven (29%) of the Dallas positive patients. Table 2 illustrates patient characteristics of these two histological subgroups. Mean right atrial pressure was higher in patients with diffuse than in those with focal myocarditis (15 ± 6 vs 7 ± 5 mmHg, P=0·002).

Frequency of cardiac antibodies

By immunofluorescence, cardiac autoantibodies were found in 13 myocarditis patients; three of them (6%) had autoantibodies of the organ-specific, five (9%) of the cross-reactive 1 and five (9%) of the cross-reactive 2 type (Table 3). Antibody titres were as follows: 1/10 in six

Table 1 Features of patients with (Dallas positive) and without (Dallas negative) biopsy-proven myocarditis

	Dallas positive (n=24)	Dallas negative (n=29)	*P*
Age at diagnosis (years)	43 ± 16	40 ± 15	ns
Male/Female ratio	14/10	21/8	ns
NYHA class I–II/III–IV	7/17	na	
Echocardiographic data	(n=24)		
LVEDD (mm)	60 ± 10	na	
Haemodynamic values; pressures (mmHg)	(n=24)	(n=4)	
RAmean	9 ± 6	10 ± 4	ns
PCW	17 ± 7	26 ± 13	ns
PAS	37 ± 11	45 ± 16	ns
PAmean	25 ± 7	na	
LVEF (%)	25 ± 10	20 ± 7	ns

LVEDD=left ventricular end-diastolic dimension; LVEF=left ventricular ejection fraction; na=not available; NYHA=New York Heart Association; RAmean=right atrial mean; PAmean=pulmonary artery mean; PAS=pulmonary artery systolic; PCW=mean pulmonary capillary wedge.

Table 2 Features of patients with diffuse and with focal myocarditis

	Diffuse (n=7)	Focal (n=17)	*P*
Age at diagnosis (years)	37 ± 11	46 ± 17	ns
Male/Female ratio	3/4	11/6	ns
NYHA class I–II/III–IV	0/7	7/10	ns
Echocardiographic data			
LVEDD (mm)	53 ± 14	63 ± 5	ns
Haemodynamic values; pressures (mmHg)			
RAmean	15 ± 6	7 ± 5	0·002
PCW	20 ± 6	16 ± 7	ns
PAS	36 ± 8	36 ± 12	ns
PAmean	28 ± 7	23 ± 7	ns
LVEF (%)	23 ± 11	25 ± 10	ns

Abbreviations as in Table 1.

sera (11%), 1/20 in three (6%) and 1/40 in four (7·5%). All positive sera contained autoantibodies of IgG class. The proportion of patients in whom antibodies of all three types were present was higher (13/53, 24%) than that observed among the ischaemic (11/186, 6%, $P=0·0001$) or the normal controls (24/270, 9%, $P=0·001$). When Dallas positive and Dallas negative patients were separately analysed, the former had higher antibody frequency (8/24, 33%) than the ischaemic (11/186, 6%, $P=0·0001$) or the normal controls (24/270, 9%, $P=0·0002$). Conversely, the finding of positive antibody status among Dallas negative patients was slightly but not significantly higher (5/29, 17%) compared to the control groups. Proportions of antibody positive patients did not significantly differ in the Dallas positive and negative groups (8/24, 33% vs 5/29, 17%, P=ns).

By ELISA, a greater proportion of myocarditis patients (9/53, 17%) had abnormally raised anti-α-myosin antibody levels compared to ischaemic (4/92, 4%, $P=0·01$) or to normal controls (4/203, 2%; $P=0·0001$). When Dallas positive and Dallas negative patients were separately analysed, Dallas positive patients had higher antibody levels than normal ($0·33 \pm 0·06$ vs $0·17 \pm 0·01$, $P=0·0005$); conversely, antibody levels among Dallas negative patients were slightly but not significantly greater compared to normal ($0·21 \pm 0·02$ vs $0·17 \pm 0·01$, P=ns) (Fig. 1). A higher proportion of Dallas positive patients had abnormal ELISA results (4/24, 17%) compared to ischaemic (4/92, 4%, $P=0·03$) or normal control subjects (4/203, 2%; $P=0·0002$). The finding of abnormal anti-α-myosin antibody results among Dallas negative patients was also higher (5/29, 17%) than in normal ($P=0·0001$) or ischaemic controls ($P=0·02$).

Antibody status and features at diagnosis

A positive result by immunofluorescence and/or an abnormal ELISA result were found in 18 myocarditis patients (34%). Mean anti-α-myosin antibody titres by ELISA were higher in patients who, by immunofluorescence, had higher titre (1/20 to 1/40) antibody compared to those who had lower titre antibody (1/10) or were antibody negative; anti-α-myosin antibody titres were $0·20 \pm 0·02$ in the 43 patients negative by immunofluorescence, $0·18 \pm 0·03$ in the six patients with antibody titre (1/10), $0·32 \pm 0·04$ in the three patients with titre 1/20 and $0·66 \pm 0·03$ in the remaining four with titre 1/40 ($P=0·001$). There were no significant associations between clinical or diagnostic features and antibody status or titre. The Dallas positive patients who had cardiac

Table 3 Cardiac antibody types by indirect immunofluorescence in myocarditis

	Organ-specific n (%)	Cross-reactive 1 n (%)	Cross-reactive 2 n (%)	Total n (%)
Myocarditis (n=53)	3 (6)	5 (9)*§§	5 (9)†§§	13 (24)‡**
Dallas positive (n=24)	2 (8)††	3 (12·5)§‡‡	3 (12·5) ‡‡	8 (33)•**
Dallas negative (n=29)	1 (3)	2 (7)	2 (7)	5 (17)
Ischaemic heart disease (n=186)	3 (1·6)	4 (2)	4 (2)	11 (6)
Normals (n=270)	7 (2·5)	8 (3)	9 (3)	24 (9)

P vs normals: **P*=0·02; †*P*=0·04; ‡*P*=0·001; §*P*=0·01; *P*=0·02; •*P*=0·0002.
P vs ischaemic heart disease: §§*P*=0·01; ***P*=0·0001; ††*P*=0·04; ‡‡*P*=0·007.

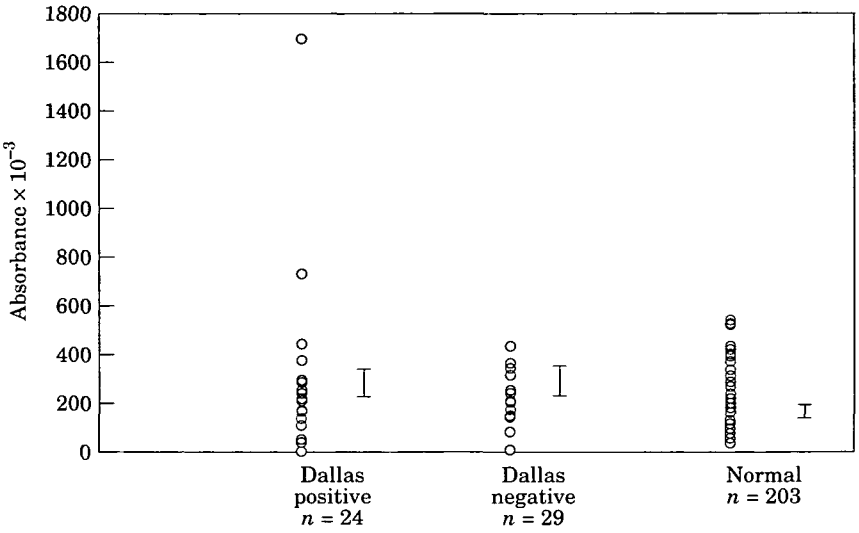

Figure 1 **Scatterplot of anti-α-myosin antibody levels by ELISA in normals and in myocarditis patients according to histological characterization. Antibody levels are given as mean ± standard error. Myocarditis patients classified as Dallas positive had higher anti-α-myosin antibody levels (0·33 ± 0·06) than normal (0·17 ± 0·01, P=0·0005). Dallas negative patients tended to have higher antibody levels (0·21 ± 0·02) than normal (P=0·08). Dallas positive and negative groups had similar antibody levels (P=ns).**

antibodies by immunofluorescence and/or ELISA (n=10) and those who were antibody negative (n=14) had similar left ventricular ejection fraction at baseline (25 ± 9 vs 24 ± 10 respectively, P=ns), at week 28 (31 ± 15 vs 36 ± 15 respectively, P=ns) and at week 52 (35 ± 17 vs 43 ± 18 respectively, P=ns).

Discussion

In this study in 34% of patients from the Myocarditis Treatment Trial we detected the same cardiac autoantibody markers that are found in dilated cardiomyopathy[8,12,13]. This provides evidence for autoimmunity in a subset of patients with myocarditis, and is consistent with myocarditis being part of the spectrum of dilated cardiomyopathy, as hypothesized on the basis of clinical observations[27,28]. As shown here in myocarditis and previously in dilated cardiomyopathy, the cardiac antibodies of the IgG class detected by immunofluorescence[8,13] or the high titre (1/320) IgG anti-α-myosin antibodies[12] are found in patients with myocarditis/dilated cardiomyopathy at higher frequencies than in disease or normal controls. This is in keeping with the findings of other autoimmune conditions[17,29,30]. Whether or not the antibodies detected by immunofluorescence[8,13] or ELISA[12] have a direct role in causing myocardial damage remains to be determined; this important issue needs to be addressed, e.g. by passive transfer experiments in susceptible animal models[17].

We employed ELISA, in addition to immunofluorescence, to screen myocarditis sera; although the two techniques have different cut-offs, antibody titres were related. This is not surprising, because in the ELISA we used purified human α-myosin, which is one of the organ-specific autoantigens recognized by the antibodies detected by immunofluorescence[10]. Another group has recently identified anti-myosin antibodies in myocarditis, but they employed ventricular β-myosin[24]. Since β-myosin is present both in human ventricles and in slow-skeletal muscle fibres[10], they detected those autoantibodies which are partially cross-reactive with skeletal muscle. Both α-(organ-specific, atrial) and β-(cross-reactive, ventricular)-myosin are recognized autoantigens in dilated cardiomyopathy[10] and in experimental autoimmune myocarditis[31]. Thus, our data and the work by Lauer[24] are complementary in showing that in human, as in murine myocarditis, both cardiac-specific and skeletal muscle cross-reactive antibodies to myosin are produced.

Our autoantibody frequency in myocarditis is lower than previously reported[9,19–23]. Potential explanations for this include the use of different techniques, antigen preparations, serum dilutions and other experimental conditions, In addition, with regard to studies which used immunofluorescence[7,9,19], lack of a unified nomenclature for the observed antibody patterns represents a major obstacle to comparing results among workers. Although indirect immunofluorescence is only semiquantitative, we used a standardized technique, that in our experience is reliable and highly reproducible[8,13]. It is hoped that introduction of standard experimental procedures and nomenclature would in the near future lead to comparison and pooling of antibody results from at least some reference centres. This goal has already been achieved world-wide for detection of other

organ-specific autoantibodies, e.g. islet cell antibodies, employing indirect immunofluorescence[29].

Other factors, besides technical pitfalls, may account for the discrepancies among groups in relation to myocarditis. Neumann *et al.*[9], using immunofluorescence, had a higher antibody frequency (59%) in myocarditis compared to us. Conversely, their results in dilated cardiomyopathy, in relation to both antibody frequency and staining pattern (diffuse cytoplasmic) are comparable to those reported by us[8]. It is likely that difficulties in patient characterization and diagnosis of myocarditis also play an important role; in addition, some reports had low patient numbers[5], some included only few patients with biopsy-proven myocarditis[5,19,20,24], or included Dallas negative patients classified as myocarditis on the basis of immunohistology[24].

Another finding of this study was that equal proportions of Dallas positive and Dallas negative patients had detectable autoantibody levels. Similarly, in the study by Lauer *et al.* where most patients were Dallas negative, a sizable proportion was autoantibody positive[24]. The lack of correlation of antibody status with the biopsy features suggests that there may be inaccuracy when diagnosis of myocarditis is made on histological criteria alone. This view is supported by the findings of several workers who have documented, by immunohistochemistry, endomyocardial biopsy features of immune activation, e.g. abnormal expression of HLA and adhesion molecules, presence of activated inflammatory cells, in Dallas negative patients with myocarditis/dilated cardiomyopathy[24,25,32,33].

Most studies, including the present work, show that the immune markers (autoantibody or immunohistological features) are found in only a proportion of patients with myocarditis; this suggests that, in the subset of negative patients, there would be some in whom myocardial damage is not autoimmune and could be mediated by infectious agents[34,35]. There are cases of chronic myocarditis which can be reasonably assumed to be post-infectious[15,16]. On the other hand, giant cell myocarditis has distinctive autoimmune features (e.g. association with other autoimmune disorders and disease recurrence in the transplanted heart)[17,36]. It is likely that infectious and autoimmune myocarditis, with or without an environmental trigger, accounts for the disease in different patient subsets[37]. This would explain the variable courses of the disease among patients, as well as inconclusive results on the response to immunosuppressive therapy in myocarditis/dilated cardiomyopathy[26,38].

In conclusion, in this study the detection of disease-specific, cardiac autoantibodies of the IgG class, using two standard immunological techniques, in sera from well-characterized patients from the Myocarditis Treatment Trial suggests an incidence of immune-mediated myocarditis of at least 30%. Proportions of antibody positive patients did not significantly differ in the Dallas positive and Dallas negative groups, suggesting that future evaluation of patients with myocarditis should incorporate immunohistology and autoantibody testing, as well as detection of viral genome by molecular techniques, to identify patients with autoimmune myocarditis (i.e. those with positive immune markers in the absence of virus) in whom immunosuppression is of potential benefit.

A.L P.C. was supported by the Veneto Region Target Project on Cardiomyopathies and the National Research Council target project 'FAT MA' (Rome, Italy), J.H.G., and K.M.B. by the British Heart Foundation. The Myocarditis Treatment Trial was supported by the National Heart, Lung and Blood Institute (RO-1-HL34744), and the National Centre for Research Resources (M01-RR00064). §See ref. [26] for a list of investigators and their institutions.

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