Heart antibodies in cardiomyopathies

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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 disease3 4 and in the post-cardiotomy state5 presumably merely reflects cardiac damage, while their presence in other disorders6 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/cardiotomy 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

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Idiopathic' pericarditis</td>
<td>3</td>
</tr>
<tr>
<td>Tuberculous pericarditis</td>
<td>1</td>
</tr>
<tr>
<td>Constrictive pericarditis</td>
<td>1</td>
</tr>
<tr>
<td>Complete heart block</td>
<td>2</td>
</tr>
<tr>
<td>Thyrotoxic heart disease</td>
<td>1</td>
</tr>
<tr>
<td>Anomalous pulmonary venous return</td>
<td>1</td>
</tr>
<tr>
<td>and constrictive pericarditis</td>
<td></td>
</tr>
<tr>
<td>Left atrial myxoma</td>
<td>1</td>
</tr>
<tr>
<td>Right atrial thymoma</td>
<td>1</td>
</tr>
<tr>
<td>Angina with normal coronary arteries</td>
<td>1</td>
</tr>
<tr>
<td>Thalassaemia major with decreased</td>
<td>1</td>
</tr>
<tr>
<td>left ventricular compliance</td>
<td></td>
</tr>
<tr>
<td>Mitral valve prolapse</td>
<td>1</td>
</tr>
<tr>
<td>Bradycardiacias</td>
<td>1</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1</td>
</tr>
</tbody>
</table>

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Sera were separated from cells immediately, divided into aliquots, and stored at $-35^\circ\text{C}$ or below. Sera (kindly supplied by Professors Goodwin and Waterson) from eight patients with idiopathic congestive cardiomyopathy attending the Hammersmith Hospital, were kept at $-20^\circ\text{C}$ 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 $-20^\circ\text{C}$. Both groups of sera were transferred to Birmingham in solid carbon dioxide and stored at $-35^\circ\text{C}$ 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 $-70^\circ\text{C}$. Six micron sections were cut on a cryostat at $-20^\circ\text{C}$ and air dried on microscope slides for 15 minutes. Though any mammalian myocardium might have been used, the baboon's was chosen as primate heart may share antigens with human heart not found in other mammals and fresh material was available. Nonmyopathic 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 OGI 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.

(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 $\chi^2$ technique incorporating the correction factor of Yates.
Table 2  *Experiment 1: single sandwich*

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. tested</th>
<th>No. positive</th>
<th>Significance of difference from controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congestive cardiomyopathy</td>
<td>8</td>
<td>3</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Alcoholic cardiomyopathy</td>
<td>6</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Control</td>
<td>27</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table 3  *Experiment 2: single sandwich*

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. tested</th>
<th>No. positive</th>
<th>Significance of difference from controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congestive cardiomyopathy</td>
<td>9</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>Alcoholic cardiomyopathy</td>
<td>9</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertensive cardiomyopathy</td>
<td>6</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertrophic cardiomyopathy</td>
<td>6</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Myocarditis</td>
<td>6</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>Post-infarct cardiotomy</td>
<td>9</td>
<td>6</td>
<td>p&lt;0.02</td>
</tr>
<tr>
<td>Miscellaneous and ischaemic heart disease</td>
<td>16</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

**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/cardiotomy 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 sera obtained from the Hammersmith Hospital.

Only post-infarct/cardiotomy 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-sarcoplastic by some authors and possibly that described as sarcolemmal-subsarcolemal by others, is the most specific for striated muscle. 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/cardiotomy 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/cardiotomy 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/cardiotomy 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.
Heart antibodies in cardiomyopathies

**Table 4 Double sandwich**

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. tested</th>
<th>+ or more</th>
<th>more than +</th>
<th>+ or more</th>
<th>more than +</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congestive cardiomyopathy</td>
<td>13</td>
<td>7</td>
<td>5</td>
<td>+NS</td>
<td>+NS</td>
</tr>
<tr>
<td>Alcoholic cardiomyopathy</td>
<td>12</td>
<td>6</td>
<td>2</td>
<td>+NS</td>
<td>+NS</td>
</tr>
<tr>
<td>Hypertensive cardiomyopathy</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>-NS</td>
<td>-NS</td>
</tr>
<tr>
<td>Hypertrophic cardiomyopathy</td>
<td>11</td>
<td>4</td>
<td>1</td>
<td>-NS</td>
<td>-NS</td>
</tr>
<tr>
<td>Myocarditis</td>
<td>7</td>
<td>3</td>
<td>3</td>
<td>+NS</td>
<td>+NS</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>+p&lt;0.05</td>
<td>+NS</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>16</td>
<td>8</td>
<td>3</td>
<td>+NS</td>
<td>+NS</td>
</tr>
<tr>
<td>Post-infarct cardiotomy</td>
<td>11</td>
<td>7</td>
<td>6</td>
<td>+p&lt;0.05</td>
<td>+p&lt;0.02</td>
</tr>
<tr>
<td>Recurrent pericarditis</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>-p&lt;0.02</td>
<td>-NS</td>
</tr>
<tr>
<td>Hammersmith congestive cardiomyopathy</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>-p&lt;0.05</td>
<td>-NS</td>
</tr>
<tr>
<td>Control</td>
<td>40</td>
<td>16</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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/cardiotomy 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/cardiotomy 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, endomysial, and intercalated disc staining to moderate intensity. Unless very intense, this fluorescence was ignored. No serum exhibited diffuse fluorescence significantly more intensely than others. Endomysial 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/cardiotomy 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 endomysial 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
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. When single tests were performed on most of these patients a negative result was seen.

Only a selection of the publications of Das and Bölte and their colleagues are listed. 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. Unlike Das, Bölte 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. 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. The report of Sack et al. 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


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